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USE OF MONOCLONAL ANTIBODIES TO STUDY
THE STRUCTURE AND FUNCTION OF NICOTINIC ACETYLCHOLINE RECEPTORS
ON ELECTRIC ORGAN AND MUSCLE AND TO DETERMINE THE STRUCTURE
OF NICOTINIC ACETYLCHOLINE RECEPTORS ON NEURONS

Annual Report

Jon M. Lindstrom

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During the past year, we made rapid progress on molecular studies of nicotinic acetylcholine receptors of both the muscle and neuronal types. Human muscle type nicotinic receptors composed of α, β, γ, and δ subunits were shown to be produced in large amounts by a cell line. These receptors were characterized electrophysiologically, immunologically, biochemically, and by cDNA sequencing. For the first time, this provides a system which produces relatively large amounts of human muscle acetylcholine receptors for biochemical, molecular genetic, and pharmacological studies. Neuronal nicotinic receptors were immunoaffinity purified from brains of several species and were found to consist of only two kinds of subunits. Multiple receptor subtypes have been found. Receptor proteins have been immunolocalized. Subunit-specific monoclonal antibodies were made which react with human neuronal nicotinic receptors. cDNAs have been identified for both the acetylcholine-binding subunit and the structural subunit of one subtype of neuronal nicotinic acetylcholine receptor. Thus, the complete primary structure of a neuronal nicotinic receptor has been established. This should lead to expression systems in which their functional properties can be conveniently studied and provide further probes for studying their function *in situ*.

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ABSTRACT

Acetylcholine receptors are of interest to the U.S. Army primarily because many chemical warfare agents act indirectly or directly through these receptors to kill by paralyzing respiratory muscles and to disrupt cognitive behavior by interaction with neuronal receptors.

Rapid progress was made during the past year on molecular studies of nicotinic acetylcholine receptors characteristic of both muscle and neurons.

In particular, a human cell line which produces large numbers of muscle nicotinic receptors was characterized in detail, including electrophysiological characterization of the receptors at the single channel level, purification of the receptor, immunological identification of its subunits, and cloning and sequencing of cDNAs for two of its four subunits. Agents were found which can up-regulate or down-regulate expression of this receptor. Thus, for the first time, this provides a convenient tissue culture system for studying human muscle nicotinic acetylcholine receptor structure and function.

We have immunoaffinity purified neuronal nicotinic receptors from the brains of chickens, rats, and cattle and developed monoclonal antibodies which react with receptors from human brain. Monoclonal antibodies have been used to localize neuronal nicotinic receptors in frogs, chickens, and rats, and human studies are in progress. Receptor subtypes have been found which share a common structural subunit. Unlike muscle nicotinic receptors, none of these receptors bind α-bungarotoxin, all have only two kinds of subunits (an acetylcholine-binding subunit and a structural subunit), and many of the nicotinic receptor subtypes have very high affinity for nicotine. Unlike muscle nicotinic receptors which function in the postsynaptic membrane, some of these neuronal nicotinic receptors are located presynaptically or extrasynaptically. We have obtained N-terminal amino acid sequences for both subunit types of receptors purified from chicken and rat brains. This information was used to identify cDNAs which code for these subunits. We have cloned and sequenced a cDNA for a structural subunit which is shared by two nicotinic receptor subtypes purified from chicken brain and may also be shared by ganglionic nicotinic receptors. We have also cloned and sequenced a cDNA for the acetylcholine-binding subunit of the receptor subtype from chicken brain which corresponds to the most frequent nicotinic receptor subtype in mammalian brain.

An α-bungarotoxin-binding protein on neurons in many cases has been shown not to be an acetylcholine-gated cation channel, yet there is reason to believe that it is a member of the nicotinic receptor gene family. We have purified the α-bungarotoxin-binding protein from brains of chickens and rats.

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We have prepared high titer antisera to it which also recognize α-bungarotoxin-binding proteins from human brain. We are in the process of making subunit-specific monoclonal antibodies to aid in identifying cDNAs for its subunits.

Thus, rapid progress has been made in molecular characterization of several members of the neuronal nicotinic receptor gene family, and the complete primary structure of one neuronal nicotinic receptor subtype has been defined.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the ''Guide for the Care and Use of Laboratory Animals,'' prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

For the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45CFR46.

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INTRODUCTION

Significance of Nicotinic Receptors

Many chemical warfare agents inhibit acetylcholinesterase and thereby affect acetylcholine receptors, first overstimulating them due to excess acetylcholine and then blocking their function by desensitization. Some of these agents may also have some direct effects on acetylcholine receptors. Acetylcholinesterase inhibitors kill primarily through their effects on muscle nicotinic receptors, resulting in paralysis of respiratory muscles. These agents may also produce long-lasting effects on brain function which are less well characterized.

Nicotinic acetylcholine receptors play a role in several medically important phenomena. Short-acting nicotinic receptor antagonists are used to block receptors on muscle during surgery to ensure muscle relaxation. An antibody-mediated autoimmune response to acetylcholine receptors in muscle is responsible for the muscular weakness characteristic of myasthenia gravis. Some insecticides can act like chemical warfare agents to threaten respiratory muscles. The venom of cobras and kraits, some frog skins and some corals, for example, contain toxins that block receptor function. Neuronal nicotinic receptors are decreased in Alzheimer's and Parkinson's diseases, although this effect may be secondary to others. Neuronal nicotinic receptors are probably involved in the addiction to nicotine and the pleasurable responses of tobacco smokers to smoking.

The roles of nicotinic receptors in normal neuromuscular transmission are understood in substantial detail and serve as an archetype for the study of other receptors and membrane channel proteins; however, the roles of nicotinic receptors in the normal function of the brain are not at all well understood. The lack of understanding of the normal functional roles of neuronal nicotinic receptors is due in part to the complexity of understanding any brain function, but is also due in substantial measure to the fact that until quite recently there have been no suitable molecular probes for these receptors.

Nicotinic Receptor Studies

Acetylcholine receptors are part of a gene family which includes muscle type acetylcholine receptors, like those of mammalian skeletal muscle or fish electric organs, as well as neuronal nicotinic receptors of several subtypes. This gene family is part of a gene superfamily of structurally related proteins evolved from common ancestors. It also includes receptors for glycine and gamma-aminobutyric acid (GABA) and may include several other ligand-gated ion channels.[1] This is summarized in Figure 1.

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During the past year, we wrote a rather extensive review on molecular studies of neuronal nicotinic acetylcholine receptors.[2] Basically, this showed that studies of neuronal nicotinic receptors, until quite recently, have lagged far behind those of muscle type receptors because neuronal systems lacked a few critical experimental advantages. Muscle type acetylcholine receptors can be obtained in relatively huge amounts from fish electric organs and can be biochemically identified, quantitated, and affinity purified using snake venom toxins like α-bungarotoxin. Neuronal nicotinic receptors are present in brain in much lower amounts, and they do not bind α-bungarotoxin. Recently, using probes developed from studies of muscle type receptors, studies of neuronal nicotinic receptors proceeded rapidly. Two kinds of probes have been used in these studies, monoclonal antibodies (mAbs) to detect receptor protein and cDNAs to detect receptor mRNAs. Both of these approaches depend on the idea that there should be substantial structural homology between nicotinic receptors from muscles and nerves. This idea has been validated by the results of these studies. Both mAb and cDNA approaches proceeded rapidly along the lines shown in Figure 2 and are now being rapidly integrated. For example, partial protein sequences of subunits of purified cDNAs were used to identify subunit cDNAs, and histological studies on rat brains localized receptor proteins in cell processes by means of mAbs, while receptor mRNAs were localized in cell bodies by means of cDNAs.

Our approach to studying nicotinic acetylcholine receptors has been to identify, purify, and characterize receptor proteins using mAbs and then use amino acid sequence data on the purified proteins to identify receptor cDNAs.

The results of studies of the structures of neuronal nicotinic receptors that we have conducted during the past 2 years are summarized in Figure 3. Acetylcholine receptors from eel electric organs are of the muscle type.[2] They are composed of 4 kinds of subunits, termed α, β, γ, and δ in order of increasing molecular weight.[3,4] The α subunits form the acetylcholine binding site, as shown by affinity labeling with 4-(N-maleimido)benzyltrimethylammonium iodide (MBTA).[4,5] We used mAb 35, which binds to α subunits of receptors from eel electric organ, to purify receptors from chicken brain.[7] These receptors had only two kinds of subunits, rather than the four kinds of subunits found in muscle receptors. We termed these subunits α and β in order of increasing molecular weight, in keeping with the convention for labeling subunits of receptors from electric organs and muscle. These receptors, unlike receptors from muscle, did not bind α-bungarotoxin, and they had much higher affinity for nicotine than did receptors from muscle.[8] The acetylcholine binding site could be affinity labeled with MBTA.[9] The acetylcholine binding site was located on the higher molecular weight of the two neuronal receptor subunits, rather than on the lowest molecular weight subunit, as in the case of receptors from muscle. This created a confusion

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in nomenclature which was exacerbated when others[10,11] trying to identify cDNAs for the subunits of neuronal nicotinic receptors by low stringency hybridization with cDNAs for the α subunits of muscle nicotinic receptors termed the homologues they identified 'α,' to indicate that they had been identified with muscle α subunit probes and to suggest that they contained the acetylcholine binding site because they contained cysteines homologous to those affinity labeled by MBTA in electric organ receptors. We showed by N-terminal amino-acid sequencing[12] (unpublished, in the case of receptors from chicken brain) that the brain nicotinic receptor subunits that were affinity labeled by MBTA (and termed 'β' in order of increasing molecular weight) corresponded to the cDNAs termed 'α.' Others subsequently identified another muscle α subunit neuronal homologue which lacked cysteines homologous to those labeled by MBTA and which could substitute in <u>Xenopus</u> oocyte expression studies for muscle β subunits.[13,14] They termed this cDNA $\beta 2$. By N-terminal amino-acid sequencing, we showed that this rat cDNA and a corresponding chicken cDNA we cloned coded for the lowest molecular weight subunit which we had termed 'α.'[15] To minimize confusion, we now refer to the MBTA-labeled subunits of neuronal nicotinic receptors as 'acetylcholine-binding subunits' and the other subunit of neuronal nicotinic receptors as 'structural subunits.' This nomenclature has the additional virtue that it can also be applied to other receptors in the ligand-gated ion channel superfamily. Thus, one could speak of the 'GABA-binding subunit' of the GABA receptor rather than the 'β' subunit (in GABA receptors, as in neuronal nicotinic receptors,[9] the higher molecular weight of the two subunits forms the transmitter binding site). Using mAbs made to receptors purified from chicken brain using mAb 35, a second nicotinic receptor subtype with low affinity for mAb 35 was discovered.[16] It was present in equal amounts and had similar pharmacological properties. It used the same low molecular weight structural subunit as did the other receptor subtype, but its acetylcholine-binding subunit was of much higher molecular weight. A similar receptor subtype comprising >90% of the high affinity nicotine binding sites in rat brain was immunoaffinity purified from rat brains.[17] This purification was done with mAb 270 to the structural subunit of chicken brains. mAb 270 also binds to the ganglionic type nicotinic receptors in rat PC12 cells.[18] These have much lower affinity for nicotine than do the receptors purified from brain.[19] If receptors from brain and ganglia share a common structural subunit, then purification of receptors from brain using a mAb to this subunit would be expected to purify both subtypes in proportion to their composition in brain. Thus, the purification results showing only acetylcholine-binding subunits of the molecular weight characteristic of brain nicotinic receptors and not ganglionic receptors suggest that in brain there is much more of the receptor subtype purified than of the ganglionic type. The acetylcholine-binding subunit of the receptor immunoaffinity purified from rat brain was shown by N-terminal amino-acid sequencing to correspond to the cDNA termed '$\alpha 4$.'[12] The structural subunit was similarly shown to

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correspond to the cDNA termed $\beta2$.[15] mAb 270 did not cross-react with receptors from the brains of cattle or humans.[20,21] However, mAbs to receptors purified from rat brain were used to identify receptors with high affinity for nicotine in the brains of cattle and humans. The receptor purified from bovine brain consisted of two subunits very similar in molecular weights to those of receptors from rat brain.[21] Receptor could not be purified free of proteolytic damage from human brain, presumably because of long intervals between death and freezing the brains.

While looking for neuronal cell lines which might express neuronal nicotinic receptors, we discovered that the human neuromedulloblastoma cell line TE671 actually expresses muscle acetylcholine receptors. This surprising result is very useful because there is no other convenient source of human muscle acetylcholine receptors for structural or functional studies. During the past year, we have made substantial progress on studies of these human muscle nicotinic receptors.[22,23] In the future, studies of cDNAs for neuronal nicotinic receptors may lead to transfected cell lines expressing neuronal receptors which will become as useful for studying these receptors as TE671 is now for studying human muscle nicotinic receptors.

MUSCLE TYPE NICOTINIC ACETYLCHOLINE RECEPTORS

Torpedo Electric Organ Nicotinic Receptors

We are mapping epitopes for antibodies using synthetic peptides according to a method developed by Dr. Mario Geysen.[24] The complete sequence of a subunits of receptors from Torpedo californica electric organ was synthesized as a series of 430 overlapping octomers (peptide 1 = amino acids 1-8, peptide 2 = amino acids 2-9, etc.). The peptides were synthesized on plastic pegs in a format which fit into 96-well microtiter plates to permit assay of antibodies bound to the peptides with peroxidase-labeled anti-antibody. After each assay the bound antibodies were removed and the peptides reused. Both synthesis and assays were monitored by computer to manage the large amount of data involved.

Figure 4 shows typical results. Part A shows the pattern obtained using rabbit antisera raised to a subunits of receptors from Torpedo. There are several prominent epitopes. The pattern is consistent with results we obtained previously by immune precipitation of synthetic peptides. Of course, on native receptor there are epitopes which depend on the native conformation of the receptor, and these will not be detected by this technique. Part B of Figure 4 shows that using an affinity column with the synthetic peptide a152-167, you can affinity purify antibodies directed at the epitopes in that sequence from rabbit antiserum to a subunits. This validates the specificity of the Geysen approach. This epitope is interesting because all antibodies raised to denatured subunits are thought to bind to the cytoplasmic surface of the receptor. If this sequence were exposed on the cytoplasmic surface, it would mean that there were at least two transmembrane domains prior to the first hydrophobic sequence in a. However, these antibodies are not adsorbed by reasonable excesses of native receptor, and these affinity-purified antibodies do not bind well to native receptor in membranes as detected by electron microscopy with colloidal gold labeling. Also, mAbs to this epitope do not bind to the native receptor. Part C of Figure 4 shows that rat antisera to a subunits detect the same basic pattern of epitopes detected by rabbit antisera to a subunits. Part D of Figure 4 shows that we have mAbs directed at several of the prominent epitopes on a subunits. We had previously mapped these mAbs to the same epitopes by immune precipitation of synthetic peptides,[25] and in some cases by peptide mapping,[26] confirming the Geysen technique. mAb 236 does not bind to native receptor, whereas mAbs 142 and 147 bind to the cytoplasmic surface.[25,26] These results show that the sequence between a350 and a370 is on the cytoplasmic surface of the native receptor.

Our goal is to use the Geysen technique to precisely map the epitopes of a number of antibodies and then determine whether these antibodies bind to the extracellular or cytoplasmic

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surface of the native receptor by colloidal gold labeling using electron microscopy and other techniques. This will help to map the transmembrane orientation of the subunit polypeptide chain.

We will synthesize the complete sequence of α subunits of receptors from human muscle and of subunits of neuronal nicotinic receptors in order to permit similar studies on these receptors, using the antisera and mAbs which we have already made.

Human Muscle Nicotinic Receptors Expressed in TE671 Cells

We first discovered that the human neuromedulloblastoma cell line TE671 expresses muscle-like acetylcholine receptor by observing that autoantibodies from myasthenia gravis patients reacted as well with these receptors as they did with receptors extracted from human legs.[27] Then we found that mAbs which recognized neuronal nicotinic receptors with high affinity for nicotine from human brain did not recognize receptors from TE671.[20] Recently we found that antisera to the α-bungarotoxin-binding protein purified from chicken brain reacted with α-bungarotoxin-binding proteins from human brain, but did not react with receptors from TE671 cells.[23] These results all contradicted the earlier idea that TE671 cells expressed receptors corresponding to the α-bungarotoxin-binding protein from human brain[28,29] and supported the idea that these cells express muscle type receptors.

During the past year, we have established that TE671 expresses large numbers of functional muscle type nicotinic receptors.[23]

Acetylcholine induced the opening of receptor channels with a conductance of 44-45 pS (**Figure 5**).[23] αBungarotoxin blocked receptor activity (**Figure 6**). A majority of the channel openings were brief (65% have a time constant of 0.82 ms) whereas a minority of the openings were more prolonged (35% have a time constant of 3.3 ms). These properties are all consistent with those of muscle nicotinic receptors.[30]

Receptors from TE671 cells were shown to be of muscle type by immune precipitation reaction with mAbs raised against receptors purified from human leg muscle, like mAbs 203 and 207.[23] These receptors were shown to be antigenically of the extrajunctional type by their ability to react with two other mAbs to receptors from human muscle, C_9 and F_8, which have been shown[31] to react only with extrajunctional receptors.

Receptors were affinity purified from TE671 cells by affinity chromatography using α-bungarotoxin (**Table I**).[23] The specific activity of unpurified receptors from TE671 is 1/20 that of <u>Torpedo</u> electric organ, twice that of mouse BC3H-1 muscle cells,[32] and 27 times that of fetal calf muscle.[33] Pure

receptor was obtained at high yield free from proteolytic degradation.

Receptors purified from TE671 cells were composed of four kinds of subunits of apparent molecular weights 42,000, 52,500, 55,000, and 62,000 (**Figure 7A**) which corresponded antigenically to the a, β, γ, and δ subunits of receptor from _Torpedo_ electric organ by Western blotting (**Figure 7B**).[23] The a subunits were shown to form the acetylcholine binding site by affinity labeling with MBTA (**Figure 7C**).

Receptors purified from TE671 cells sedimented on sucrose gradients as monomers and dimers similar in size to those from _Torpedo_ electric organ (**Figure 8A**).[23] Muscle type receptors from sources other than _Torpedo_ electric organ are not usually found as dimers. This could be because there are no dimers in most muscles or because these dimers are either selectively no-extracted or not proteolyzed when they are extracted. _Torpedo_ dimers are formed by a disulfide bond between their δ subunits.[34] However, although reduction did dissociate dimers of TE671 receptor (**Figure 8B**), we found no evidence of δ dimers (**Figure 8C**). Thus, these human muscle receptors appear to be noncovalently dimerized by a mechanism that is influenced by disulfide bond reduction.

TE671 cells contain poly (A^+) mRNAs corresponding to the a, β, γ, and δ subunits of receptors from mouse muscle (**Figure 7D**).[23]

The sequence of a cDNA for the a subunit of receptor from TE671 is identical to that expected from a human genomic clone and exhibits ≥95% sequence identity with a subunits from murine and bovine muscle (**Figures 9 and 10**).[22,23] This unequivocally identifies TE671 a subunits as being of the muscle type. It further confirms the remarkable degree of sequence conservation characteristic of muscle nicotinic receptor a subunits. Many sequences are conserved including a glycosylation site, four hydrophobic domains, and cysteines at 192, 193 which in _Torpedo_ are known to be disulfide linked[35] and which are near the acetylcholine binding site and can be affinity labeled by MBTA after reduction of this disulfide bond [6]

The sequence of a cDNA for the δ subunit of receptor from TE671 is that expected for δ subunits of a muscle nicotinic receptor (**Figures 11 and 12**).[23] It exhibits ≥90% sequence identity with δ subunits from murine and bovine muscle. The sequences of δ subunits are less well conserved than are those of a subunits. The greatest extent of dissimilarity is in the sequences between amino acids δ398-451. This region is expected to be on the cytoplasmic surface.[25,26] The sequence of δ subunits and a subunits from TE671 exhibits 30% identity, showing that the subunits are homologous, as expected.[36] The δ subunits from TE671 show conservation of four hydrophobic domains, three putative N-glycosylation sites, and three

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putative phosphorylation sites. The δ subunits of receptors from TE671, like those of muscles from other sources, lack the penultimate C-terminal cysteine through which δ subunits from Torpedo[37] are thought to dimerize (Figure 12).

Expression of nicotinic receptors in TE671 cells can be regulated by neurotransmitters, hormones, and neurotrophic factors (Figure 13).[23]

Nicotine, dexamethasone, and human calcitonin gene-related peptide (hCGRP) increase the amount of receptor. hCGRP reduces the functional ability of the receptors it induces (Figure 13). This could occur by a mechanism like phosphorylation which has been shown to decrease receptor function by speeding desensitization.[38,39] Dexamethasone increased subunit mRNAs, but hCGRP and nicotine did not. Clearly, up-regulation of receptors in TE671 cells appears to occur by several mechanisms. Both transcriptional regulation and other mechanisms are involved. The mechanisms by which receptor synthesis is up-regulated in TE671 cells and in muscle cells may differ because, whereas nicotine up-regulates the amount of receptor in TE671 cells, agonists down-regulate the amount of receptor in muscle cells.[40] Regulation may also differ between neuronal cell types, since we have observed that agonists up-regulate the amount of receptor in rat PC12 cells, whereas in chicken ciliary ganglion culture, agonists down-regulate the amount of receptor.[41] The effects of CGRP are especially interesting in view of its postulated role as a neurotrophic factor in chicken muscle, where it increases receptor expression via an increase in cyclic AMP concentration.[42,43]

Treatment of TE671 cells with forskolin down-regulates the amount of receptor to negligible levels (Figure 13). Forskolin is an adenylate cyclase activator[44] reported to induce muscle receptor phosphorylation and thereby enhance desensitization.[45-47] In TE671 cells, forskolin down-regulates the amount of receptor subunit mRNAs. It also inhibits cell division and causes the cells to send out neuron-like processes (Figure 14). Cell death was not noted. It does not induce the appearance of neuronal nicotinic receptors detectable by binding of specific mAbs or [^{3}H]nicotine. If the effects of forskolin are mediated by an increase in cyclic AMP, then the increase in receptor induced by CGRP must not be mediated by increased cyclic AMP.

The physiological significance of expression of muscle type nicotinic receptors in TE671 cerebellar neuromedulloblastoma cells is unknown. We have considered three possibilities.[23] Expression of muscle AChR could be an aberration induced by the transformation events which produced this tumor line. Another possibility is that TE671 cells may derive from a neuronal cell type which transiently expresses muscle type receptors during development. This would explain why muscle type receptors are not detected in extracts of adult human brains. One could imagine that a developmental inducer normally affects the

development of this neuronal cell type in the same way that forskolin affects TE671 cells, terminating receptor synthesis and cell division. A third possibility is that TE671 cells derive from a rare cell type normally present in adult cerebellum. It has been reported that there are interneurons in rat cerebellar cortex with receptors that can be activated by nicotine and blocked by α-bungarotoxin.[48]

NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS

Frog Brain

Dr. Peter Sargent, a collaborator at the University of California, Riverside, tested 42 mAbs to receptors from electric organs, which were known to bind to receptor in frog muscle,[49] for their ability to cross-react with neuronal nicotinic receptors in the optic tectum of the frog _Rana pipiens_.[50] Twenty-eight of these were shown to bind by immunoperoxidase cytochemistry to a part of the neuropile corresponding to a subset of retinotectal projections. Removal of one retina caused loss of labeling in the contralateral tectum. Thus, in frogs, as we have previously observed in goldfish,[51] chickens,[52] and rats,[52] there are nicotinic receptors on the processes of retinal ganglion cells. In these studies localization of the labeled receptors by electron microscopy revealed that there were receptors in extrasynaptic locations. This is the highest resolution of neuronal nicotinic receptor location to date and is very provocative in showing that these receptors may not be directly presynaptic, but also extrasynaptic, where their functional role may be more nearly as a paracrine receptor than as a synaptic receptor. Presynaptic nicotinic receptors may be involved in the regulation of process outgrowth and synapse formation.[53,54] Other workers have found evidence for presynaptic neuronal nicotinic receptors,[55,56] and a substantial, but as yet unknown, fraction of neuronal nicotinic receptors may not function in the postsynaptic role of nicotinic receptors in ciliary ganglia[57] or muscle.

Avian Brain and Retina

Drs. Kent Keyser and Harvey Karten, collaborators at the University of California, San Diego, used two mAbs which bind to nicotinic receptors from chicken brain to identify and characterize cholinoceptive neurons in chick retina.[58] Using indirect immunofluorescence gave excellent cell level resolution. Some amacrine cells, displaced ganglion cells, and about 15% of ganglion cells were labeled. The retina is a very interesting system to pursue because of the evidence for nicotinic receptors on retinal ganglion cells in every species we have examined,[50-52,58] and because the retina provides a very well defined and accessible bit of neuronal architecture. Future studies with mAbs specific for subtypes of neuronal nicotinic receptors may help us understand the functional roles of the subtypes. Also, we will be conducting _in situ_ hybridization to localize the mRNAs as well as the proteins for the receptor subunits within the architecture of the retina.

Drs. James Watson and Tom Podleski, collaborators at Cornell University, used two ^{125}I-labeled mAbs to localize neuronal nicotinic receptor subtypes with respect to ^{125}I-α-bungarotoxin binding sites in brains of zebra finches.[59] Their primary

interest was in the sexually dimorphic song nuclei, which, it turns out, lack substantial amounts of mAb binding. However, a by-product of these studies was mapping the receptors with respect to 65 brain areas. mAb 270 to structural subunits was used to map all nicotinic receptor subtypes sharing this subunit. mAb 35 was used to map the receptor subtype with the lower molecular weight acetylcholine-binding subunit (see Figure 3). As expected, mAb 35 labeled a subset of the areas labeled by mAb 270. However, in two small areas, labeling with mAb 35 occurred in the absence of labeling with mAb 270. Muscle type receptors would meet this criterion, except these two areas did not bind ^{125}I-α-bungarotoxin, as muscle receptor would. Thus, this either reveals an artifactual cross-reaction of mAb 35 or reveals another minor nicotinic receptor subtype in avian brain.

In collaboration with Dr. Larry Swanson at The Salk Institute, we localized receptors in the optic tectum of chicken brain with ^{125}I-mAb 270 and showed that removing retinal ganglion cells, by removing an eye, removed labeling in the areas of the optic tectum to which the ganglion cells project.[52] Labeling in other layers was preserved. Five years ago, we first detected mAb 35 binding in the spiriform nucleus and its projections to the optic tectum.[60] These projections were not affected by eye removal.

The structural subunit used by both subtypes of nicotinic receptors that we had immunoaffinity purified from chicken brains was purified and the N-terminal amino acid sequence determined[15] (Figure 15). This sequence was identical in the structural subunit of the receptor purified from rat brain, suggesting, as expected, that they are closely related proteins. The N-terminal amino acid sequence permitted identification of a cDNA clone which coded for the structural subunit of receptors from chick brain[15] (Figures 16 and 17).

cDNAs for the structural subunit were screened for by low stringency cross-hybridization. A partial sequence of a chicken cDNA termed $\gamma 2$, which had homology with receptors from muscle but lacked cysteines corresponding to Torpedo $\alpha 192, 193$, had been reported.[61] Using an oligonucleotide based on this sequence, cDNAs were tentatively identified. The library was also screened for hybridization with a cocktail of probes for the rat cDNAs $\alpha 2$,[51] $\alpha 3$,[10] and $\alpha 4$[11] including probes for $\alpha 3$ and $\alpha 4$ obtained from Drs. Steve Heinemann and Jim Boulter. The cDNAs $\alpha 2$, $\alpha 3$, and $\alpha 4$ are all muscle nicotinic receptor α subunit homologous, containing homologues of the cysteines $\alpha 192,193$ which were identified from various rat neuronal tissues by low stringency hybridization. Clones which were positive in both screens were assayed on Northern blots of chick brain poly (A^+) RNA in order to detect those which gave patterns distinct from $\alpha 2$, $\alpha 3$, and $\alpha 4$. This lead to the clone described in Figures 16 and 17.

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Like the other nicotinic receptor subunits identified to date,[10,11,61,62] the structural subunit has a leader sequence and four hydrophobic domains and shows conservation of several amino acid sequences with other subunits, not including cysteines corresponding to a192, 193.[15] It is least conserved in the putative cytoplasmic domain. This subunit corresponds to the rat brain cDNA β2.[14]

In the course of screening for the structural subunit, we also identified the cDNA for the acetylcholine-binding subunit of the receptor subtype in chicken brain with the 75,000 molecular weight acetylcholine-binding subunit (see Figure 3). Sequencing of this cDNA shows that it is similar to the rat brain cDNA termed a4[11] and identical to the chicken brain cDNA termed a4.[63] It was identified using the sequence of the N-terminal amino acid sequence determined for the high molecular weight acetylcholine-binding subunit purified from chicken brain. It has cysteines corresponding to a192, 193 and a unique very long putative cytoplasmic domain.

Thus, we now have sequenced cDNAs for both subunits of one subtype of nicotinic receptor which we have purified from chicken brain. We are beginning expression studies in Xenopus oocytes using these complete cDNAs. Using specific probe sequences from them we are beginning in situ hybridization studies in chicken retina with Kent Keyser and Harvey Karten to complement our studies of the localization of the receptor subunit proteins in these cells using mAbs. The retinal ganglion cells, for example, should contain mRNAs in their cell bodies to code for subunits of the receptors which they transport in great numbers to their axonal terminations in the optic tectum. Also, we will be determining whether this structural subunit is precisely the sequence expressed in the receptors of chicken ciliary ganglia, or whether these are similar, immunologically cross-reactive, but distinct proteins.

Rat Brain

By determining the N-terminal amino acid sequence of the acetylcholine-binding subunits of nicotinic receptors purified from rat brain (Figure 15), we[12] were able to identify the rat brain cDNA a4 as corresponding to this subunit. This cDNA was sequenced by Jim Patrick and coworkers.[11] Its sequence is shown in Figure 17. It contains cysteines corresponding to Torpedo a192, 193, which was expected from our observation that after reduction with dithiothreitol, this subunit was specifically affinity labeled with MBTA.[9] This subunit contains a very large unique putative cytoplasmic domain, which accounts for the high molecular weight of this subunit. This domain may be exposed on the outer surface of vesicles of newly synthesized receptor and interact with the transport mechanism that moves these receptors to their distant localization in, for example, the central processes of retinal ganglion cells. It is also the cytoplasmic domain of receptors that might interact with extrinsic membrane

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proteins and the cytoskeleton to localize receptors specifically at synaptic or extrasynaptic locations. The cytoplasmic surface might also be susceptible to phosphorylation and other regulatory interactions.

Boulter et al.[13,14] have shown that injection of _Xenopus_ oocytes with the cDNAs $\alpha 4$ and $\beta 2$ produces functional receptors. This is consistent with our observations that nicotinic receptors from brain actually have only two kinds of subunits,[7,16,17,21] and that their subunit sequences correspond to these cDNAs.[12,15]

Do neuronal nicotinic receptors composed of only two kinds of subunits preserve the fivefold symmetry[64] of the $\alpha 2\beta\gamma\delta$ subunit composition of receptors from muscle, or do they have a simpler $\alpha 2\beta 2$ subunit structure? We know that these receptors exist as monomers.[7,16,17,21] Also, we know that they have at least two subunits of each kind.[16] But the small amounts of receptor have made it very difficult to determine whether there are precisely two or three copies of each subunit.[12,16] An $\alpha 2\beta 2$ stoichiometry is appealing because it avoids homosubunit interactions that are inevitable with five subunits of two kinds[2] (see Figure 18). Now that we know the N-terminal amino acid sequences of both subunit types in a neuronal nicotinic receptor subtype,[12,15] it should be possible to definitively determine stoichiometry by N-terminal sequencing whole receptor and determining the ratio of yields for amino acids from each subunit. If the subunit composition is $\alpha 2\beta 2$, yields will average 1:1, whereas they will average 1.5:1 in favor of one subunit if there is fivefold symmetry.

Nicotinic receptors were located throughout rat brains using ^{125}I-mAb 270 to the structural subunit of receptors from chicken brain.[52] This should localize all receptor subtypes which share this structural subunit. Results are summarized in Figure 19. In general, receptor localization closely paralleled that obtained previously using [3H]nicotine.[65] There was evidence for extrasynaptic receptors on some sensory neurons and for pre- or extrasynaptic receptors in several areas. For example, retinal ganglion cells were shown to transport receptors to their axon terminals in the superior colliculus, and removal of an eye eliminated receptors in the contralateral superior colliculus. It seems clear that neuronal nicotinic receptors function both postsynaptically and pre- or extrasynaptically and it will be interesting to try to determine these functional roles in detail and associate them with particular receptor subtypes.

A library of nine mAbs was made[21] to nicotinic receptors purified from rat brains as described in Table II. This included mAbs for both the acetylcholine-binding subunit and the structural subunit. It will be instructive to use these for immunohistological localization of receptors as well.

Bovine Brain

Using mAbs raised against nicotinic receptors from rat brain, it was possible to characterize and purify nicotinic receptors from bovine brain.[21] Like the nicotine receptors from brains of chickens[7,16] and rats,[17] these receptors do not bind a-bungarotoxin, and they have high affinity for nicotine (Table III). Unlike nicotinic receptors of the ganglionic type from bovine chromaffin cells,[66] they do not bind mAb 35. This suggests that receptors on chromaffin cells differ by at least one subunit from those in brain. Immunoaffinity purified receptors from bovine brain are composed of a 50,600 apparent molecular weight structural subunit and a 74,400 apparent molecular weight acetylcholine-binding subunit[21] (Figure 20). Like nicotinic receptors from chicken and rat brain, these receptors exist as monomers slightly larger than those of receptor from Torpedo (Figure 21). This suggests that nicotinic receptors in the brains of all mammals will be quite similar and the predominant subtype will have this basic structure.

Human Brain

Nicotinic receptors in human brain were distinguishable immunologically and pharmacologically from those in muscle (Figure 22).[20,21] Receptors with high affinity for nicotine were identified in detergent extracts of human brain using mAbs raised to receptors from rat brain (Figure 3).[21] Their pharmacological properties were similar to those of nicotinic receptors from bovine brain (Figure 23 and Table III). Like bovine receptors, they could be affinity labeled with both bromoacetylcholine (BAC) and MBTA (Figure 24) and sedimented as monomers on sucrose gradients (Figure 21). Receptors immunoaffinity purified from human brain showed signs of proteolytic degradation, presumably due to the prolonged interval between death and freezing the brain. However, all evidence suggests that their structures are very similar to those of the receptors purified from the brains of rats and cattle.

We have sent ^{125}I-labeled mAbs to Dr. Christer Kohler at Astra Alab in Soedertalje, Sweden, and to Dr. Paul Clarke at McGill University, Montreal, Canada, in order to enable them to localize these receptors in brains of monkeys and humans.

Thus, we have accumulated a substantial amount of information about the pharmacological, immunochemical, and biochemical properties of nicotinic receptors from human brain, and expect that the predominant receptor subtype will closely resemble those in other mammalian brains.

METHODS

Geysen Epitope Mapping

The Geysen[24] epitope mapping system was purchased from Cambridge Research Biochemicals (Atlantic Beach, NY) and used in conjunction with an IBM-PC and a Titertech (McLean, VA) MCC/340 Plate Reader, according to the manufacturer's directions. The sequence of Torpedo a subunits reported by Noda et al.[67] was used to synthesize a as a set of overlapping octomers. Bound antibodies were detected with commercial (CalBiochem, San Diego, CA) peroxidase-labeled goat anti-rabbit IgG at 1/10,000 dilution or with homemade peroxidase-labeled affinity purified goat anti-rat IgG or the peroxidase-labeled mouse mAb to rat IgG MAR 18.5.[68]

Culturing TE671 Cells

Cultures were grown at 37°C in 90% air 10% CO_2 in Iscove's modified Dulbecco's modified essential medium (DMEM) Irvine Laboratories (Irvine, CA) supplemented with either 10% fetal bovine serum or 5% bovine calf serum. For electrophysiological studies, 10^4 cells were plated per well in a 24-well plate on 12 mm diameter glasscover slips in medium with 10% serum. One day later, serum was reduced to 0.01%, and 2 mM liters of glutamine, 10 μg/ml insulin, and transferrin were added. Electrophysiological studies were done in 115 mM NaCl, 5 mM CaCl, 1 mM $MgCl_2$, 25 mM glucose, 25 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), pH 7.4, 10 mM tetra·thylammonium chloride, and 0.1 mM anthracene-9-carboxylic acid.

Electrical Recordings from TE671 Cells

Single channel current electrical recordings were performed in the laboratory of Dr. Mauricio Montal at the University of California, San Diego according to methods described in detail.[69] Recordings were obtained in both the cell-attached and the excised-patch configurations. The pipettes were fabricated from KOVAR glass (Corning 7052, inner diameter=1.1 mm, 70 mm long) using a vertical pipette puller (David Kopf 700C, Tujunga, CA). The pipettes were coated with Sylgard-180 (Dow Corning) within 40 μm from the tip and fire-polished immediately before use, under 320X magnification. The tip size was adjusted to yield 5-15 Mohms of open pipette resistance when filled and immersed in the buffer described before. The patch pipettes contained the indicated concentration of acetylcholine diluted in the same solution. The cells were observed with an inverted microscope (Nikon-diaphot) using a 40X objective (LWD DL 40XC, Nikon) equipped with Hoffman modulation contrast optics (Modulation Optics, Greenvale, NY). The microscope was mounted on a vibration isolation table (Micro g Technical Mfg. Corp., Waltham, MA).

A commercially available extracellular patch clamp system was used (LM EPC-5, List Electronics, Darmstadt, West Germany and Medical Systems Corp., NY). The headstage of the amplifier was mounted on a hydraulic micromanipulator (MO-10?N, Narishige, Japan). The signal output from the clamp was recorded on FM tape (Racal 4DS, Hythe, Southhampton, England, bandwidth DC to 5 kHz). All the records were filtered at 2 kHz on an 8-pole Bassel low pass filter (Frequency Devices, 9028LPF, Haverhill, MA). The data were digitized at the sampling frequency of 10 kHz in an Indec-L-11/73-70 microcomputer system (Indec, Sunnyvale, CA). Conductance levels were discriminated as described previously. Histograms of dwell times in the open state and closed states of the receptor channel were analyzed as described in detail previously.[70-72] The results of at least five different experiments in each condition are presented. All experiments were done at room temperature ($22^{\circ}C$).

Preparation of Solubilized TE671 Membrane Extracts

TE671 cell cultures were grown in T-flasks for 6 days and then expanded to 2 liter (850 cm^2) roller bottles in 5% fetal calf serum in Iscove's modified DMEM (Irvine Laboratories) with 2.5 μM dexamethasone. After 10 days in culture, the cells were harvested after aspiration of media by first rinsing with cold phosphate-buffered saline (PBS), pH 7.5, containing 10 mM iodoacetamide, 10 mM aminobenzamidine, and 1 mM phenylmethylsul-fonylfluoride (PMSF) to remove the excess media, and secondly by shaking in 25 ml per bottle of 50 mM Tris, 150 mM NaCl, 100 mM KF, 5 mM EDTA, 5 mM ethylene glycol bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 5 mM iodoacetamide, 5 mM aminobenzamidine, 0.5 mM PMSF, bestatin (10 μg/ml), Trasylol (10 μg/ml), and soybean trypsin inhibitor (10 μg/ml), pH 7.5 (buffer A). The bottles were then rinsed with four volumes of buffer A to remove any remaining cells. The cells were then pelleted by centrifugation at 3000 x g for 30 minutes. The resulting cell pellet was resuspended in 400 ml of buffer A, homogenized, and centrifuged as described in the previous step. The resulting pellet was then extracted for 30 minutes in four volumes of buffer A with 1% Thesit detergent (Boehringer, Mannheim, Indianapolis, IN) and 0.05% sodium dodecyl sulfate (SDS), pH 7.5, centrifuged at 140,000 x g for 30 minutes, and the clarified supernatant retained.

Purification of the TE671 Nicotinic Receptor

aBungarotoxin was first coupled to Sepharose CL-4B at 5.0 mg of protein/ml of gel by a modified procedure of Kohn and Wilchek.[73] The clarified, solubilized TE671 membrane extract (75-100 ml) from, typically, 12 roller bottles was applied to a 20 ml column of Sepharose CL-4B to adsorb any proteins which may nonspecifically adsorb to the affinity matrix. The eluate was then applied to a 1 ml column of α-bungarotoxin-affinity gel and both columns were washed with 150 ml of the extraction buffer.

The affinity column was consecutively washed with 200 ml of buffer A containing 1.0 M NaCl, 0.5% Thesit, and 0.05% SDS, pH 7.5, followed by 150 ml of 10 mM Tris, 0.1% Thesit, 1 mM NaN$_3$, 10 mM KF, 1 mM iodoacetamide, 1 mM aminobenzamidine, 1 mM EDTA, and 1 mM EGTA, pH 7.5 (buffer B). The affinity column was then coupled to a hydroxylapatite (HPT) column (1 ml) and the TE671 receptor eluted onto the HPT column by recirculating through both columns for 12 hours 10 ml of buffer B containing 200 mM carbamylcholine, using a peristaltic pump. After displacement of the bound protein, the HPT column was washed with 200 ml of buffer B and then eluted with 150 mM sodium phosphate, 0.5% Thesit, 1 mM NaN$_3$, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 1 M aminobenzamidine, and 1 mM iodoacetamide at pH 7.5.

Affinity Labeling of TE671 Receptor

TE671 receptor was immobilized on a-bungarotoxin-Sepharose and then affinity labeled with [^{3}H]MBTA (a gift from Dr. Mark McNamee) as previously described.[9]

Sucrose Gradient Centrifugation of TE671 Receptor

Aliquots (100 μl) of purified TE671 receptor, crude detergent-solubilized TE671 receptor, human muscle extracts, and affinity purified Torpedo receptor were incubated overnight at 4°C with 0.5 nM ^{125}I-a-bungarotoxin. These were layered onto 4.9 ml sucrose gradients (5-20% w/v in 20 mM phosphate, pH 7.5, 1.0 mM NaN$_3$, 0.5 mM PMSF, 1 mM aminobenzamidine, and 0.1% Thesit), and centrifuged for 70 minutes at 65,000 rpm at 4°C in a Beckman (Fullerton, CA) VTi65 rotor. Fourteen-drop fractions were subsequently collected from the bottom of the tubes, and radioactivity was determined by gamma counting. Reduction and realkylation of purified TE671 receptor were performed as described by Chang and Bock.[34]

Electrophoresis

Electrophoresis was conducted on acrylamide slab gels in SDS using the Laemmli[74] discontinuous buffer system. Polyacrylamide gels were silver stained for protein according to the method of Oakley et al.[75] Polyacrylamide gels of radiolabeled protein were autoradiographed for 4-24 hours at -70°C using preflashed Kodak (Rochester, NY) X-Omat-AR film and an intensifying screen. Autoradiograms were standardized by using Sigma (St. Louis, MO) prestained low molecular weight standards resolved on the same gel. Electrophoretic transfer of proteins from gels to diazophenylthioether (DPT) paper and subsequent probing with antibodies were as described previously.[76] After being probed, bound antibodies were detected by incubation with 0.5 nM ^{125}I-labeled mouse anti-rat IgG (1-3x10^{18}cpm/mol) and autoradiography.

Cloning and Sequencing of TE671 Receptor *a* Subunit cDNA

cDNA was synthesized by the RNase H method,[77] following the protocol of Watson and Jackson.[78] cDNA >1 kilobase (kb) was ligated into the cloning vector λ-zap (Stratagene, San Diego, CA). Approximately 10^5 recombinants were screened at high stringency with the 430-base pair (bp) PstI fragment of pMARa15,[79] containing the sequences for the N-terminal of the mouse muscle nicotinic receptor *a* subunit. Positive clones were plaque purified, and inset bearing plasmids were obtained using a helper phage following the supplier's protocol. Plasmid DNA was characterized by restriction enzyme digestion, followed by agarose gel electrophoresis and Southern blotting.

For DNA sequencing, nested deletions were produced by the EXO III/mung bean protocol (Stratagene) in both orientations. DNA sequencing was performed using a modification of the dideoxynucleotide chain termination method of Sanger et al.[80]

Cloning and Sequencing of TE671 Receptor *δ* Subunit cDNA

A cDNA library was prepared as previously described.[22] The filters were screened under high stringency with the ~450-bp EcoR1-Ava I fragment of cDNA clone BMD451 (a gift of Dr. Jim Boulter, The Salk Institute) coding for the 114 N-terminal amino acids of the mouse muscle acetylcholine receptor *δ* subunit. A single positive clone was identified. Plasmid DNA was characterized by restriction enzyme digestion, followed by agarose gel electrophoresis and southern blot analysis. From the ~3-kb insert, the 5' ~1860-bp Eco-Ava fragment was subcloned into a second λ-Zap vector. Nested deletions were produced by the Exo III/mung bean protocol provided by Stratagene. DNA sequencing was performed using a modification of the dideoxynucleotide chain termination method of Sanger et al.[80]

Regulation of TE671 Receptor Expression

Cultured cells grown in T flasks were harvested and 1×10^5 cells were plated in 6-well tissue culture dishes in Iscove's medium containing 10% fetal calf serum. After 2 days, the media was removed and replaced with Iscove's medium containing 10% fetal calf serum with the indicated concentrations of forskolin, nicotine, hCGRP (a gift from Dr. Jean Rivier, The Salk Institute) or dexamethasone. Forskolin and dexamethasone were dissolved in 95% ethanol while CGRP was dissolved in PBS. Ethanol or PBS alone had no effect on cell growth or receptor expression. The cells were grown for 2 days and the number of *a*-bungarotoxin binding sites, receptor function, and RNA encoding the *a*, *β*, *γ*, *δ* subunits for the TE671 receptor were determined.

The number of *a*-bungarotoxin binding sites was determined as follows. After 2 days, the medium was removed and the cells were washed 3 times with 2 ml of Iscove's media. The cells were

labeled for 1 hour with 0.5 ml of 20 nM ^{125}I-α-bungarotoxin in Iscove's medium at $37^{\circ}C$. Nonspecific binding was determined by performing the experiments as described in the presence of 1 mM carbamylcholine. After 1 hour, the cells were again washed 3 times with 2 ml Iscove's medium. The cells were solubilized with 1.5 ml of 0.5 N NaOH, removed, and bound ^{125}I-α-bungarotoxin determined by gamma counting.

Receptor function was measured by carbamylcholine-induced influx of $^{86}Rb^+$ using a modified procedure of Robinson and McGee.[81] Briefly, after 2 days of growth in the presence or absence of the various indicated effectors, the media was removed and the cells washed 3 times with 2 ml Iscove's. After the third wash, the cells were incubated for 1 hour in 0.5 ml Iscove's to allow recovery from desensitization of receptors by the effectors. Media was removed and the cells washed 2 times with 2 ml 0.5 M sucrose, 5 mM KCl, 10 mM glucose, 1.8 mM $CaCl_2$, and 15 mM HEPES, pH 7.4. The cells were then washed with 0.5 ml of the same buffer with 2 mM ouabain for 20 seconds to inhibit Na^+-K^+ ATPases. The buffer was removed and $^{86}Rb^+$ uptake was initiated by exposing cells to 0.5 ml of the ouabain buffer containing 5 $\mu Ci/ml$ of $^{86}Rb^+$ with 1 mM carbamylcholine. Control experiments were performed as described, in the absence of carbamylcholine. Uptake was terminated after 30 seconds by aspirating the radioactive solution and rapidly washing three times with 3 ml of 0.3 M NaCl, 5 mM KCl, 1.8 nM $CaCl_2$, 10 mM glucose, and 15 mM HEPES, pH 7.5. The washed cells were solubilized with 1.5 ml 0.5 N NaOH to permit $^{86}Rb^+$ and protein determination. Radioactivity was determined by liquid scintillation counting of the solubilized cells. Results were normalized as described for the determination of α-bungarotoxin binding sites.

RNA levels were measured after isolation of total RNA by the guanidine thiocyanate-CsCl procedure of Chirgwin et al.[82] The amount of RNA isolated was quantitated by OD_{260} and equal amounts of RNA from each treatment were size-fractionated by agarose gel electrophoresis containing formaldehyde. The gel was transferred to nylon membranes and probed with cloned cDNA inserts (gifts from Dr. Jim Boulter, The Salk Institute) for the α, β, and γ subunits of the mouse muscle receptor,[83] and the δ subunit probe was derived from the human cDNA clone for δ. Hybridization was conducted under highly stringent conditions: $42^{\circ}C$, 50% formamide, 5xSSPE, final washing at $65^{\circ}C$, 0.3xSSPE (where 5xSSPE is 0.9 M NaCl, 0.5 mM Na phosphate, pH 7.4, 5 mM EDTA). Autoradiography was performed as described above.

Poly (A^+) RNA was prepared from total RNA by oligo(dT) column chromatography. The mRNA species for α, β, γ, and δ were identified as described above using mouse muscle cDNA probes for each subunit.

Purification of Receptors from Brains of Rats and Chickens

Nicotinic receptors from rat brain were immunoaffinity purified using mAb 270, as has been previously described.[17] Receptors from chicken brain were purified by affinity chromatography upon mAb 270 and the receptor subtype with a 75,000 molecular weight acetylcholine-binding subunit was isolated by a second round of immunoaffinity purification using mAb 299, which is specific for this acetylcholine-binding subunit.[16] Purified receptors (~60 pmol) were resolved into subunits by electrophoresis in a 10% polyacrylamide gel and electroblotted onto quaternary ammonium derivatized glass fiber sheets.[84] Protein bands were located by fluorescent staining, excised, and subjected to gas phase microsequencing[85] upon an Applied Biosystems (Foster City, CA) model 470A protein sequencer.

Cloning and Sequencing Nicotinic Receptor Subunit cDNAs From Chicken Brain

Standard procedures were carried out as described previously.[22] Total cellular brain RNA was purified from day 17 chick embryos (E17) by the guanidinium isothiocyanate/cesium chloride method. Poly (A$^+$) RNA was obtained through two rounds of oligo(dT) chromatography. cDNA was synthesized by the RNase H method.[78] cDNA > 1 kb was ligated into the cloning vector λ-Zap (Stratagene). Clones were screened with a cocktail of probes for the nicotinic receptor family of the rat: the subcloned EcoR1 insert of the λ clone (a gift of Dr. Steve Heinemann, The Salk Institute) harboring the α4-1 gene,[11] the α3 gene[10] (λPCA48, a gift of Dr. Jim Boulter, The Salk Institute), and the insert of clone pR11, which contains fragments of the α2 gene.[61] Low stringency temperature and salt conditions were used: hybridization at 58°C in 5xSSPE (1xSSPE=0.18 M NaCl, 0.01 M NaPO$_4$, pH 7.4, 1 mM EDTA), followed by washing at 60°C in 5xSSPE and at room temperature in 0.3xSSPE. The filters were also screened with a 30mer oligonucleotide derived from the short published sequence of η_2.[61] Hybridization at 42°C in 5xSSPE, 40% formamide was followed by final washings at 60°C in 1xSSPE.

A subset of clones which were positive in both screening revealed overlapping restriction maps. Two of them, clones pCh20.2 and pCh23.1, were analyzed by DNA sequencing.[80] Both strands of pCh20.2 were sequenced throughout. pCh23.1 was sequenced completely in one orientation.

Immunocytochemistry on Chicken and Rat Brains

One-month-old white Leghorn chickens, adult Sprague-Dawley rats, and adult Balb-C mice were used in these experiments. The animals were decapitated and tissue was removed and frozen with liquid nitrogen. Cryostat sections (20 μm thick) were thaw-mounted onto slides and desiccated at 0-4°C under vacuum

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overnight. For autoradiographic localization, the sections were
overlaid with 4 nM ^{125}I-mAb 270 (radioiodinated to a specific
activity of 1-2x10^{18}cpm/mol by a modified chloramine-T method)
in 100 mM NaCl, 10 mM Na phosphate buffer, pH 7.5, 10 mM NaN$_3$,
10% normal rat serum, and 5% Carnation dried milk, and were
incubated overnight at 4°C. The slides were transferred to
Coplin jars and rinsed 5X over 30 minutes with 100 mM NaCl,
10 mM Na phosphate buffer, pH 7.5, and 10 mM NaN$_3$ at room
temperature. The sections were further rinsed in three changes
of buffer over 3 hours on a rocking platform at 4°C. They were
then dried at 37°C, mounted on cardboard in groups of 20,
overlaid with an 8x10-inch sheet of Kodak XAR5. The sections
were then postfixed in 10% formalin, dehydrated in ethanol,
defatted in xylene, rehydrated and air dried, dipped in Kodak
NTB-2 emulsion, exposed for 4 days, and developed as described
elsewhere.[86] For indirect immunofluorescence, sections were
obtained as described above, and the mAbs were localized with
goat anti-rat IgG conjugated with fluorescein isothiocyanate, as
described elsewhere.[87]

Purification of Nicotinic Receptors from Bovine Brain

Receptors from bovine brain were purified by immunoaffinity
chromatography using mAb 295 (see below) coupled to AFC resin
(New Brunswick Scientific, Edison, NJ). Briefly, a bovine brain
(obtained from a local abattoir and stored at -70°C) was
pulverized into small pieces while still frozen and then 150- to
200-g amounts were homogenized and the membranes isolated
exactly as previously described.[7] The membranes were then
extracted for 2 hours at 4°C in one volume of 2% Triton X-100 in
50 mM Tris, pH 7.2, 1 mM EGTA, 1 mM EDTA, 5 mM iodoacetamide,
5 mM benzamidine, and 2 mM PMSF. The extract was centrifuged at
140,000 x g for 1 hour in a Beckman Ti50.2 rotor and the clear
supernatant collected and then gently shaken for 15 hours at 4°C
with 3 ml mAb 295-AFC resin (6.7 mg protein/ml resin). The
resin was then loaded into a 10-ml column and washed succes-
sively with approximately 200 ml of 10 mM Na phosphate, pH 7.5,
100 mM NaCl (PBS) containing 0.5% Triton X-100, 100 ml of 10 nM
Na phosphate, pH 7.5, 1 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5%
Triton X-100, and finally with 20 ml of 20 mM Tris, pH 7.5,
100 mM NaCl, and 0.1% Thesit detergent (CalBiochem, San Diego,
CA). Bound receptor was eluted with two-column volumes of AFC
elution medium (New Brunswick Scientific) and dialyzed against 4
liters of 20 mM Tris, pH 7.5, 100 mM NaCl, and 0.05% Thesit.

Purified receptor was radioiodinated as previously
described[7] by the lactoperoxidase-glucose oxidase method
(BioRad, Richmond, CA) and kept at 4°C in PBS, 0.5% Triton X-
100, containing 1 mM Na azide and 10 mg/ml β-lactoglobulin.

Preparation of mAbs to Receptors from Rat Brains

Female Lewis rats (6-8 weeks old) were immunized with
immunoaffinity purified rat brain receptors,[17] both intact and

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denatured in SDS, emulsified in 100-200 μl of complete Freund's adjuvant (CFA). The rat that gave rise to the mAbs described was immunized intraperitoneally on day 1 with 10 pmol of receptor in CFA, on day 17 with 5 pmol of receptor (SDS denatured) in CFA, and on day 31 with 45 pmol of receptor (half of which was SDS denatured). Five days later, the rat was sacrificed and its spleen cells fused with the mouse myeloma cell line S194 15.XX0.BU1, using 50% polyethylene glycol 4000 (Merck), as described in detail elsewhere.[88] Culture supernatants were screened for binding to ^{125}I-labeled rat brain receptor by indirect immunoprecipitation using goat anti-rat IgG. Supernatants that were positive (greater than 2X background binding) upon retesting, and that upon subsequent rescreening exhibited binding to [3H]nicotine-labeled receptors in detergent extracts of chicken brain (see below) were selected for recloning. Hybridoma cells were cloned directly in agarose and then grown in bulk in Iscove's medium containing 1% fetal calf serum. Supernatants from mass cultures were concentrated to ~300 ml using a Millipore (Bedford, MA) Minitan concentrator and the immunoglobulin fraction isolated by precipitation with 18% sodium sulfate and then dialyzed against PBS containing 10 mM sodium azide.

mAb 295 was purified by chromatography upon S-Sepharose (Pharmacia, Piscataway, NJ). Briefly, 7 ml of concentrated mAb 295 was dialyzed against 50 mM morpholine ethanesulfonic acid, pH 6, 5 mM NaCl, and then applied to a 0.5x10 cm column of S-Sepharose. Bound mAb was eluted with the same buffer containing 1 M NaCl; 127 mg of mAb was recovered.

[3H]Nicotine Binding Assay

Antibody binding to receptors in crude detergent extracts of brain was determined as described previously.[8] Briefly, detergent extract (200-600 μl) was shaken gently for 15 hours at 4°C with 100 μl of culture supernatant or an appropriate amount of mAb, or serum, and 20-30 μl of a 1:1 slurry of goat anti-rat IgG Sepharose (8-12.5 mg IgG/ml gel). After washing with 2x1 ml of PBS containing 0.5% Triton X-100, the aliquots were incubated for 15 minutes at room temperature in 50 μl of 20 nM [3H]nicotine (DL-[N-methyl 3H]nicotine, specific activity 68.6 Ci/mmol, obtained from New England Nuclear, Boston, MA) in the same buffer, and then rapidly washed at 4°C with 4x1 ml of ice-cold PBS, 0.5% Triton X-100 by resuspending in the buffer and centrifuging for 20 seconds at 10,000 x g in a microfuge. Bound protein was then eluted by incubating the gel for 15 minutes with 100 μl of 2.5% SDS, 5% β-mercaptoethanol, and then sampling into 5 ml of scintillant (5% Biosolve [Beckman], 4% Liquifluor [New England Nuclear] in toluene). Radioactivity was determined by scintillation counting. Specific binding was determined by subtraction of binding in the absence of antibody.

Human Brain Tissue

Human brain tissue, primarily from patients with Alzheimer's disease, was obtained through the generosity of Dr. Robert Terry at the University of California, San Diego.

TABLE I

Purification of AChR From 20 g (12 Roller Bottles) of TE671 Cells

Fraction	Volume (ml)	Protein (mg)	AChR α-Bgt[a] Binding Sites (pmol)	(%)	^{125}I-α-Bgt Specific Activity (pmol/mg protein)
Initial extract	100	570	1,804	100	3.16
Unbound to α-Bgt affinity column	100	380	702	39	
Wash steps	650	180	342	19	
Affinity column eluate	6	0.100	776	43	7,800

[a] α-Bungarotoxin.

TABLE II

Properties of mAbs to Rat Brain Nicotinic Receptors

mAb	Titer to Receptors From Rat Brain (μM)	Cross-reaction With Brain Receptors of			Subunit Specificity	
		Chicken	Cattle	Humans	Western Blot	By Other Criteria
290	26.9	+	+	+	?	Structural
291	1.1	+	-	-	?	Structural
292	9.4	-	-	-	ACh binding	
293	4.4	+	-	+	ACh binding	
294	1.5	-	-	-	?	
295	0.5	+	+	+	?	Structural
297	1.0	+	+	+	?	Structural
298	0.7	+	+	+	?	Structural
299	31.2	+	-	+	ACh binding	ACh binding

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TABLE III

Inhibition of (DL)[^{3}H]Nicotine Binding
To Bovine and Human Brain Nicotinic Receptors

Ligand	K_I (M) Bovine Brain	K_I (M) Human Brain
Cytisine	4.2×10^{-9}	1.1×10^{-9}
(L)Nicotine	1.6×10^{-8}	6.5×10^{-9}
Acetylcholine	4.3×10^{-8}	2.7×10^{-9}
Carbachol	2.1×10^{-7}	4.1×10^{-7}
Curare	1.9×10^{-5}	4.7×10^{-5}
α-Bungarotoxin	$>10^{-6}$	$>10^{-6}$
Mecamylamine	No data	$>10^{-3}$
Hexamethonium	No data	$>10^{-3}$

Figure 1. Proteins of the ligand-gated receptor superfamily.
This newly recognized superfamily has been reviewed in 1987, from the perspective of the nicotinic receptor, by Lindstrom et al.,[2] and from the perspective of the GABA receptor by Barnard et al.[1] Note that these receptors are structurally related in terms of certain specific conserved amino acid sequences and the general conserved feature of multiple homologous subunits presumably organized like barrel staves around a central ion channel. These receptors differ not only in the transmitters they bind, but even whether the channels admit cations or anions. These changes apparently can be accomplished by relatively minor amino acid sequence changes around the ligand binding site and channel lumen. The GABA receptor is acted on at different sites by benzodiazepines, barbiturates, convulsants, anesthetics, and ethanol in addition to GABA agonists and antagonists. However, nicotinic receptors are also affected by barbiturates, channel-blocking drugs and toxins, anesthetics, and ethanol in addition to agonists and antagonists. Reproduced from reference 89.

Primordial receptor homopolymer

	Muscle AChR	Brain AChR	Ganglion AChR	α-Bgt-Binding Protein	Glycine R	GABA R	?R
Subunits	$\alpha\beta\gamma\delta(\epsilon)$	$\alpha\beta(\beta')$	$\alpha\beta$	$\alpha\beta\gamma\delta$	$\alpha\beta$	$\alpha\beta$	?
Ligand	ACh	ACh	ACh	?	glycine	GABA	?
Channel	cation	cation	cation	?	anion	anion	?
Cysteine α192,193	in ACh-binding subunit	in ACh-binding subunit	in ACh-binding subunit	in ACh-binding subunit	no	no	?
Cysteine α128,142	yes	yes	yes	probably	yes	yes	probably
Hydrophobic sequences M1,M2,M3,M4	yes	yes	yes	probably	yes	yes	probably

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Figure 2. mAb and cDNA approaches to studying neuronal nicotinic acetylcholine receptors. Note the generally parallel and often synergistic nature of these approaches. Reproduced from reference 2.

START		
	with purified AChR from electric organs	
	MAKE PROBES	
mAbs		cDNAs
to native AChR and subunits		using sequence probes
	SCREEN	
	on other species	
	SELECT	
AChRs		cDNAs
by immunoaffinity chromatography		by crosshybridization
from chicken		with vertebrate muscle
	MAKE BETTER PROBES	
mAbs		cDNAs
to chicken neuronal AChRs		to muscle AChRs
	SCREEN	
	on other species or tissues	
	SELECT	
rat AChRs		rat cDNAs
	CHARACTERIZE AChRs	

Location	Immunohistochemistry.	In situ hybridization.
Structure	Protein sequence. Subunit structure and stoichiometry. Transmembrane orientation. Posttranslational modifications. Clone cDNAs using mAbs	Nucleic acid sequence. Genomic structure Make mAbs using synthetic or expressed peptides.
Function	Ligand binding of immunoisolated AChRs. mAbs to effect function in vivo.	Electrophysiology and pharmacology of AChRs expressed in oocytes. In vitro mutagenesis.
Synthesis	Translation and assembly.	Transcriptional regulation.
Disease	Changes in AChR amount. Autoantibodies.	Genetic variants.

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Figure 3. Using mAbs to study neuronal nicotinic acetylcholine receptors from different species. mAb 35[90] to receptors from eel electric organ[4] was used to identify receptors in chicken neurons.[60] Acetylcholine-binding subunits of each receptor were identified by affinity labeling with MBTA and are marked with an *. Then receptors were immunoaffinity purified from chicken brain using mAb 35.[7,16] In turn, mAbs[16] to receptors purified from chicken brain were used to identify receptors in rat brain,[17] and mAbs to receptors in rat brain were used on cattle and humans.[21] Note that the structure of muscle acetylcholine receptors is highly conserved, by contrast with that of neuronal nicotinic receptors. For example, muscle type receptor from _Torpedo_ electric organ and human muscle both have four subunits of similar molecular weights;[23] and, despite the more than 400 million years of evolution which separate these species, the acetylcholine-binding subunits of these two receptors have 80% sequence homology.[91] By contrast, in chicken brains[16] there are equal amounts of two receptor subtypes whose acetylcholine-binding subunits differ in molecular weight by 16,000 and in mammalian brains[17,21] only one of these subtypes is observed. Reproduced from reference 2.

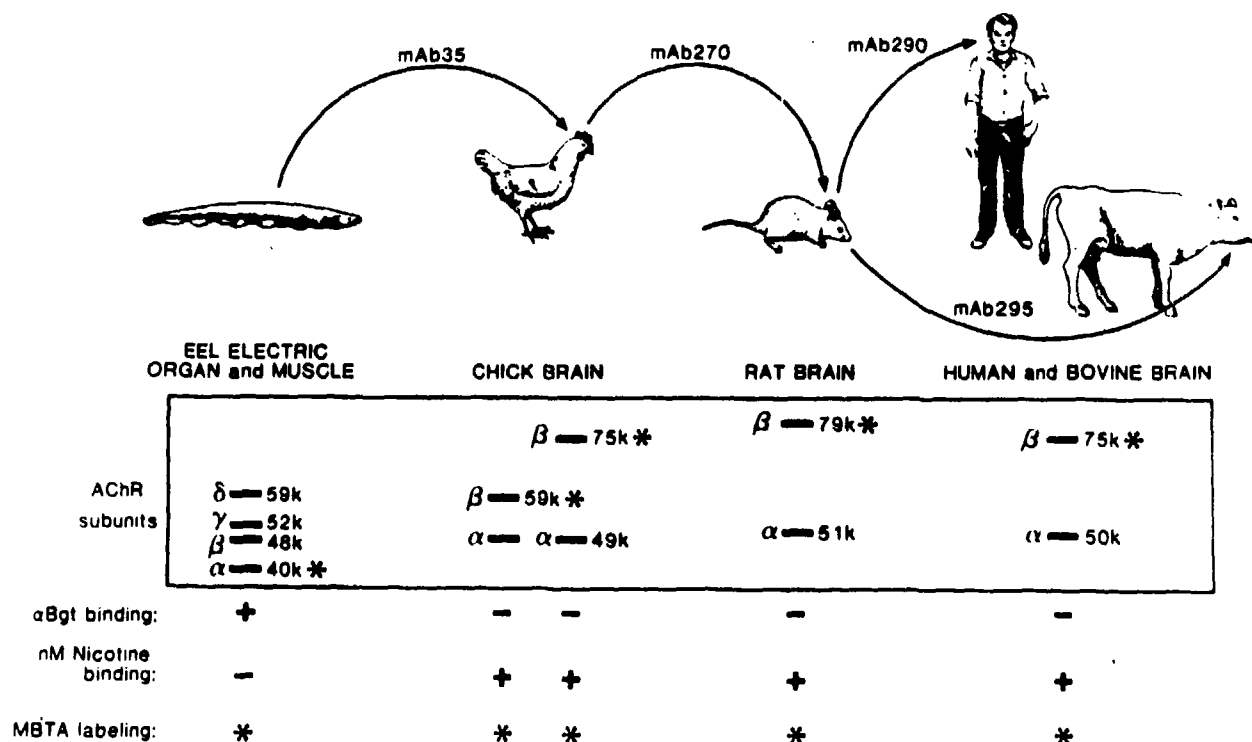|	EEL ELECTRIC ORGAN and MUSCLE	CHICK BRAIN	RAT BRAIN	HUMAN and BOVINE BRAIN
AChR subunits	δ—59k γ—52k β—48k α—40k*	β—75k* β—59k* α— α—49k	β—79k* α—51k	β—75k* α—50k
αBgt binding:	+	− −	−	−
nM Nicotine binding:	−	+ +	+	+
MBTA labeling:	*	* *	*	*

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Figure 4. Mapping epitopes on *a* subunits of receptors from *Torpedo* using the Geysen[24] synthetic peptide technique.
As described in the text, the indicated antisera, affinity purified antibodies, and cocktail of mAbs were assayed in solid phase using peroxidase-labeled anti-antibody for binding to 430 overlapping synthetic peptide octomers corresponding to the complete sequence of *a* subunits from receptors from *Torpedo* californica.
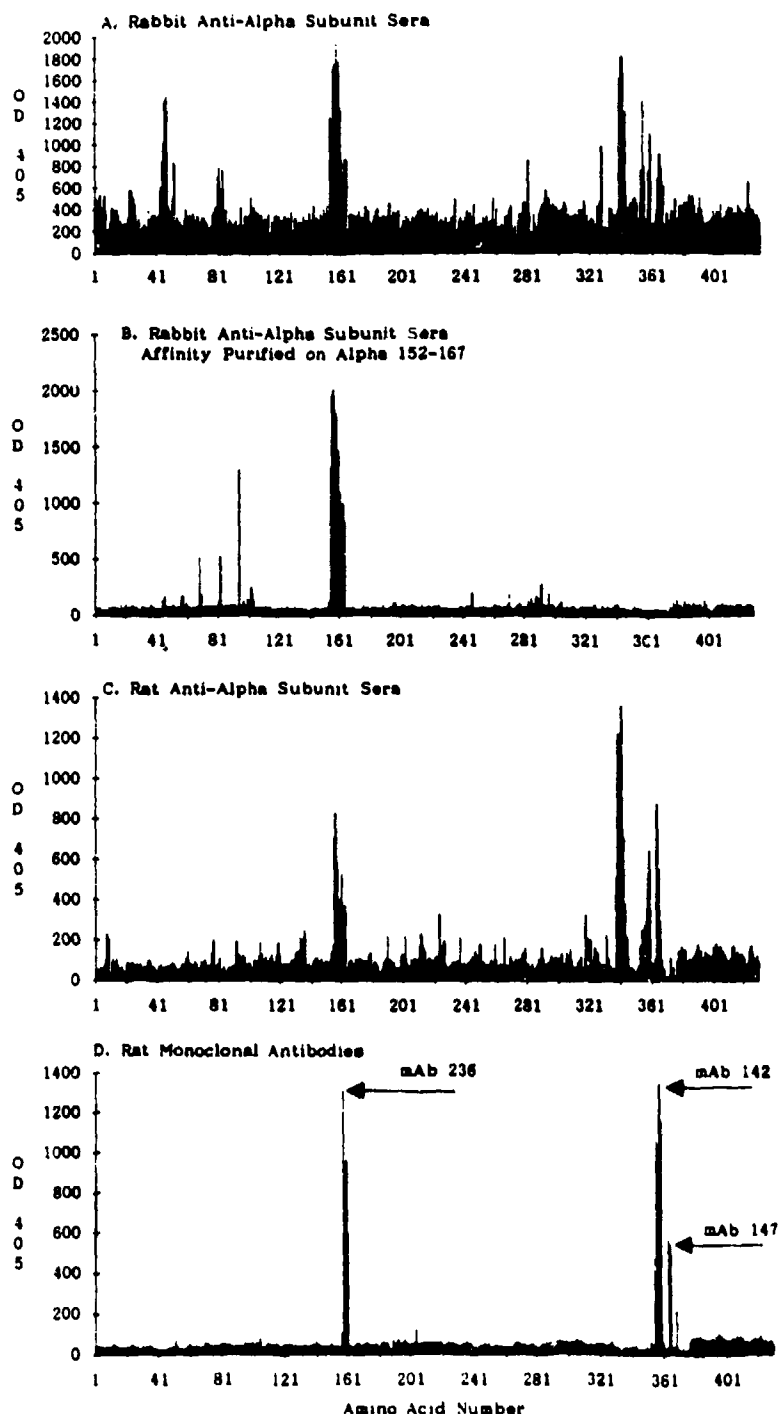
Figure 5. Acetylcholine induces openings of single receptor channels in TE671 cells. Openings were recorded at an applied voltage of 100 mv at 0.5 µM acetylcholine or 70 mv at 10 µM acetylcholine. Reproduced from reference 23.
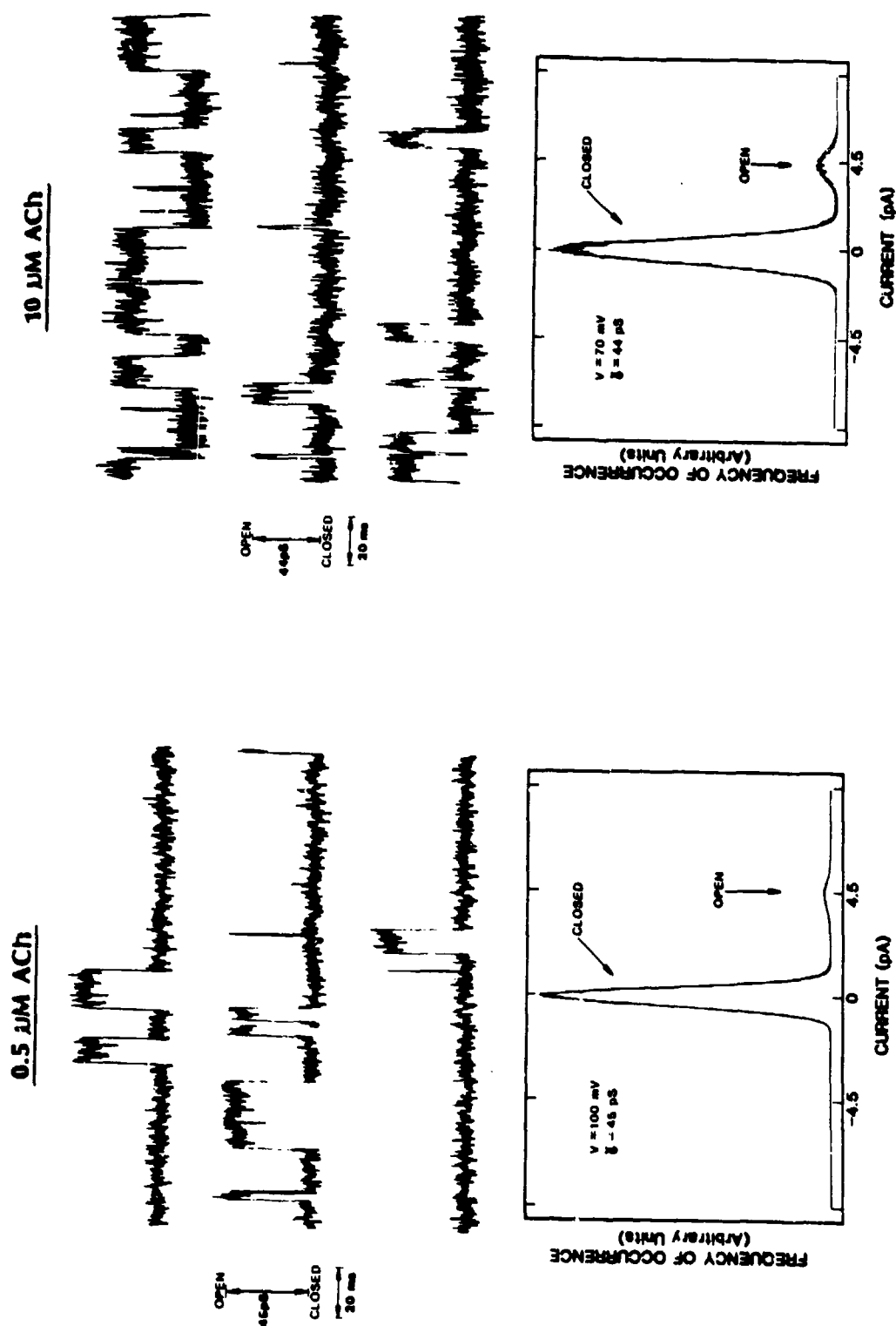
Figure 6. Analysis of single acetylcholine receptor currents in TE671 cells. A, α-bungarotoxin (0.04-0.15 μM in various experiments) blocks the opening of receptor channels induced by acetylcholine (at 0.5-50 μM). B, The receptor channels behave ohmically with a conductance of 45 pS. C, Receptor channels frequently open for short durations (0.82 ms) and less frequently open for longer durations (3.3 ms) in 10 μM acetylcholine at 100 mv. The data for 2,035 openings (noisy curve) are fit by a sum of two exponentials (smooth curve). Reproduced from reference 23.
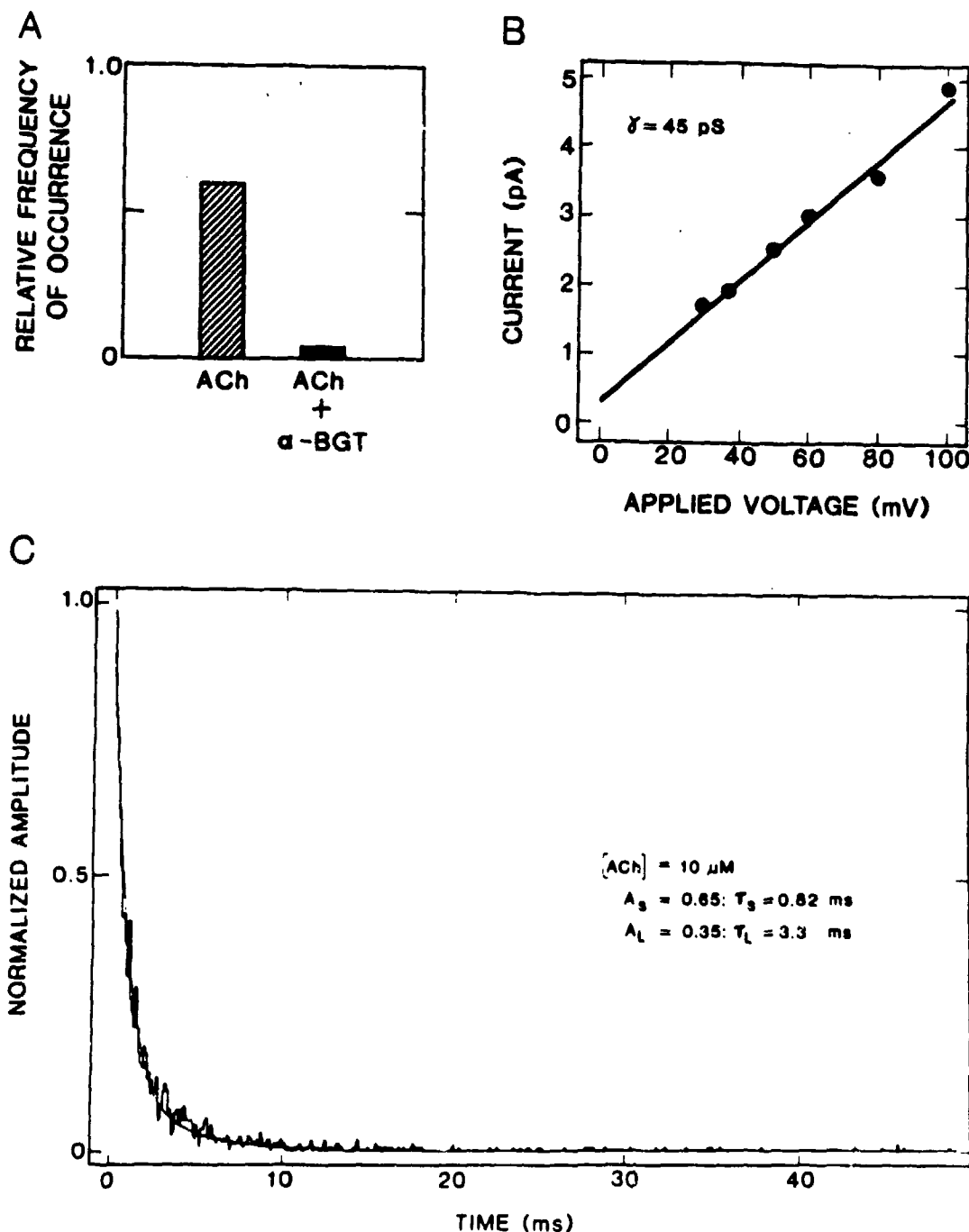

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Figure 7. Subunits of receptors from TE671 cells. A, Receptors purified from TE671 and <u>Torpedo</u> electric organ have similar molecular weights as shown by electrophoresis under reducing conditions on acrylamide gel in SDS and staining with Coomassie blue. B, Subunits from TE671 receptors correspond to those of receptor from <u>Torpedo</u> by western blotting. Bound antibodies were localized by autoradiography using ^{125}I-mouse anti-rat IgG. C, Affinity labeling with [^{3}H]MBTA and specific inhibition of labeling by carbamylcholine identify the a subunit of receptor from TE671 as forming the acetylcholine binding site. D, Poly (A$^+$) mRNAs for the four subunits of receptor from TE671 are detected by high stringency hybridization using cDNAs for mouse muscle receptor a, β, γ, and δ subunits. The cDNA probes used were described by Heinemann et al.[83] Reproduced from reference 23.

45
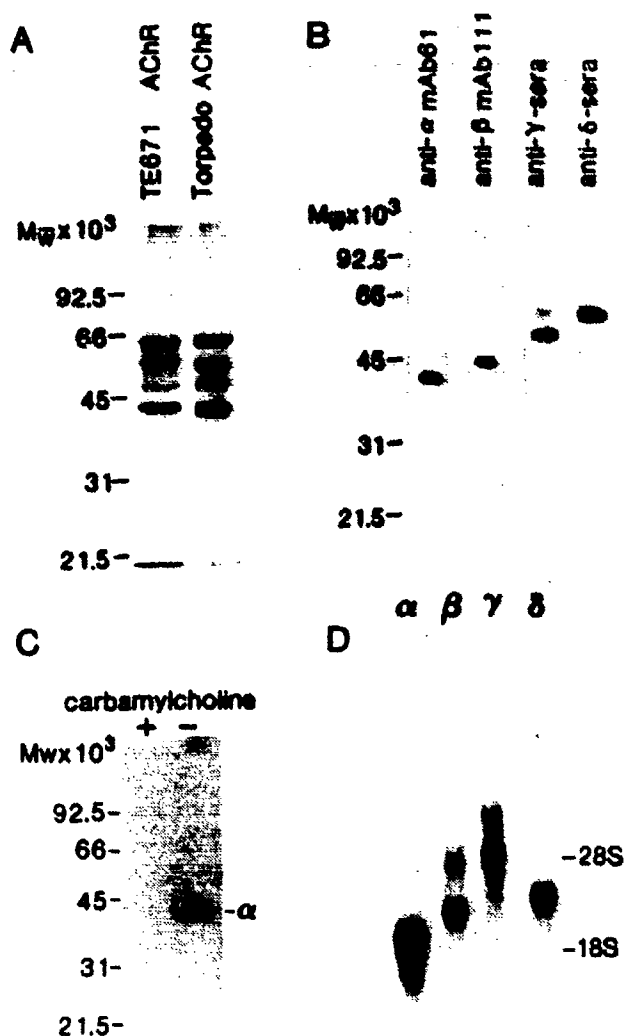
Figure 8. Nicotinic receptors from TE671 cells consist of both monomers and dimers. A, Sucrose gradient centrifugation resolves monomers and dimers of ^{125}I-α-bungarotoxin-labeled receptors. B, Reduction and alkylation reduce the amount of TE671 dimers seen on sucrose gradients. C, Electrophoresis on acrylamide gels in SDS under nonreducing conditions reveals no dimerized subunits in TE671 receptor dimers. Reproduced from reference 23.
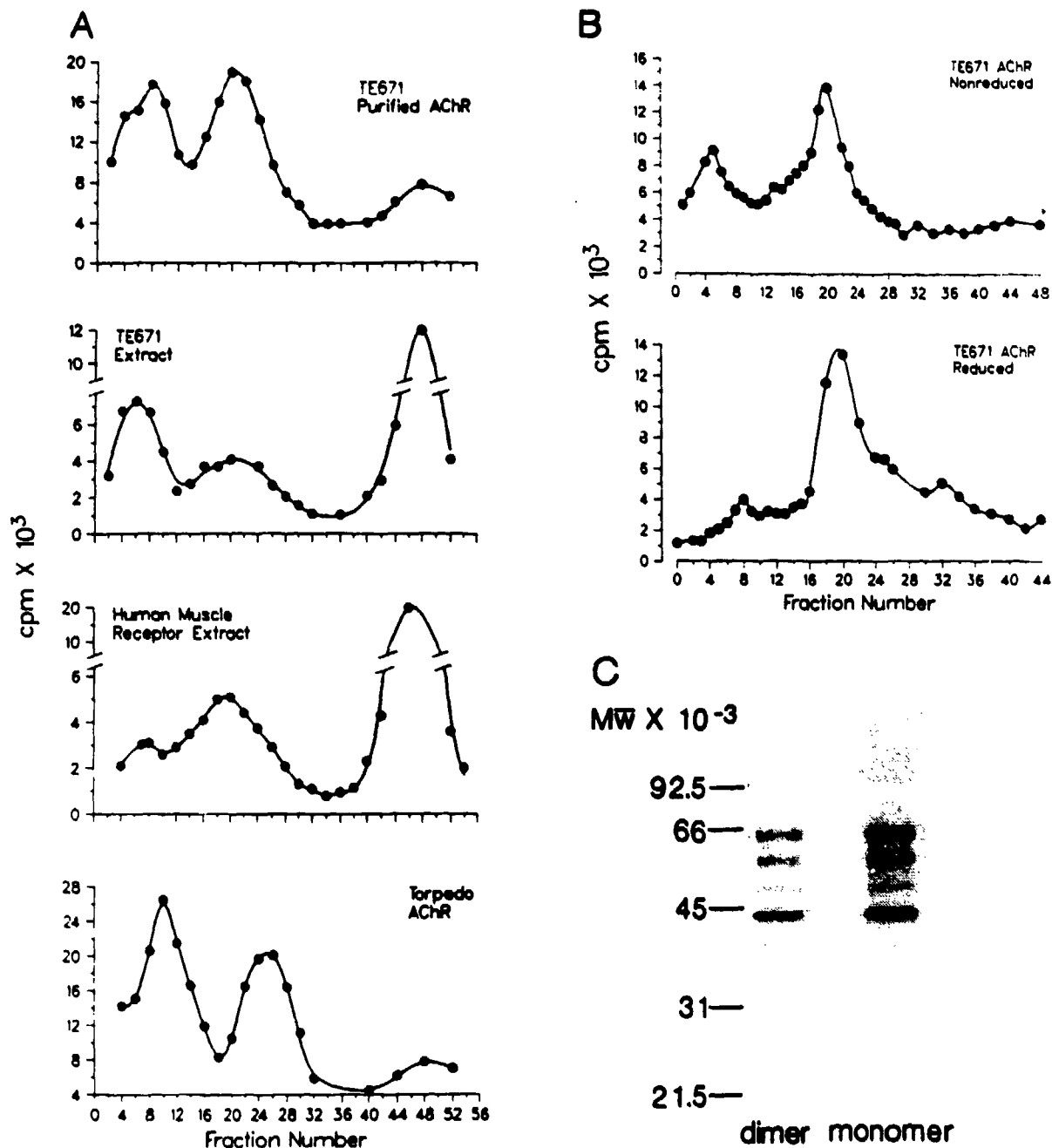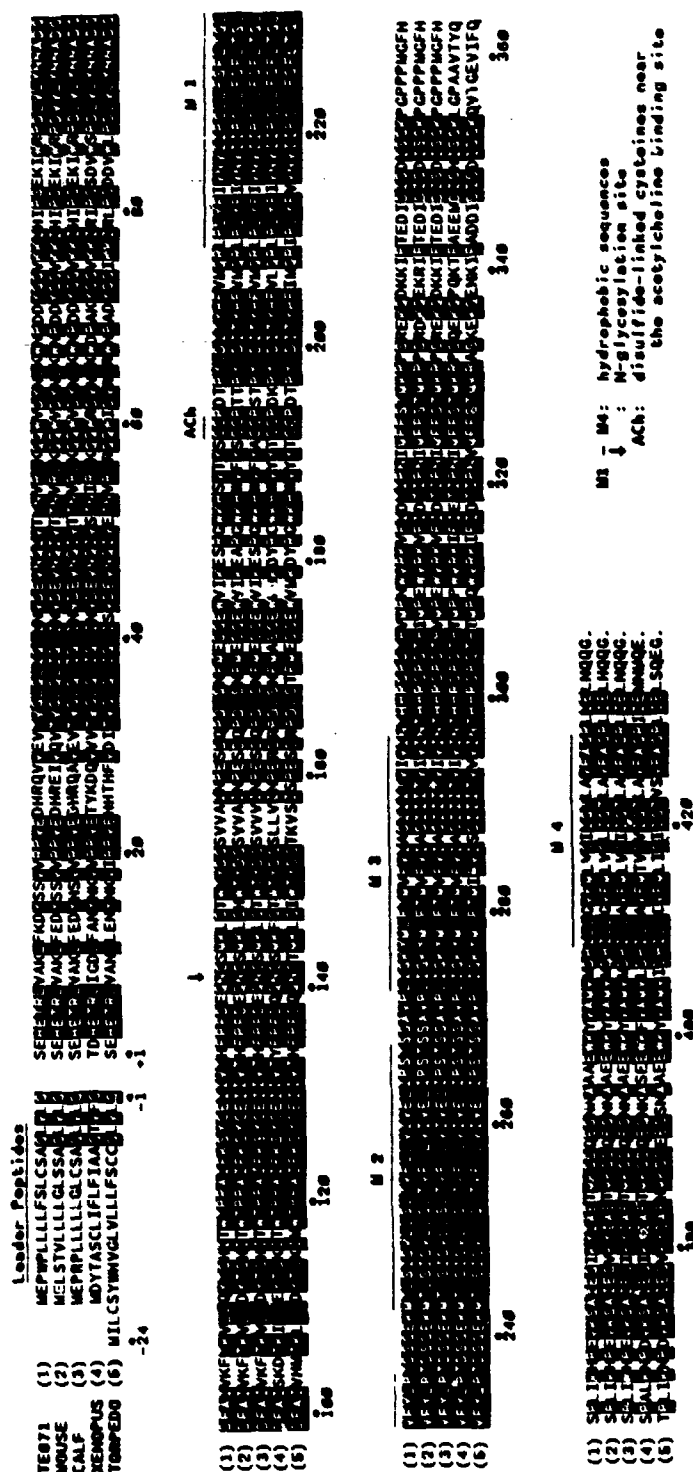
Figure 9. Nucleic acid sequence and deduced amino acid sequence of a cDNA for the α subunits of receptors from TE671 cells. Reproduced from reference 22.

```
                                                    -20
                                           MetGluProTrpProLeuLeuLeuLeuPheSerLeuCysSer
AAGCACAGGCCACCACTCTGCCCTGGTCCACACAAGCTCCGGTAGCCCATGGAGCCCTGGCCTCTCCTCCTGCTCTTTAGCCTTTGCTCA
      -100            -80            -60            -40            -20
        -1  1
AlaGlyLeuValLeuGlySerGluHisGluThrArgLeuValAlaLysLeuPheLysAspTyrSerSerValValArgProValGluAsp
GCTGGCCTCGTCCTGGGCTCCGAACATGAGACCCGTCTGGTGGCAAAGCTATTTAAAGACTACAGCAGCGTGGTGCGGCCAGTGGAAGAC
        -1  1            20            40            60
HisArgGlnValValGluValThrValGlyLeuGlnLeuIleGlnLeuIleAsnValAspGluValAsnGlnIleValThrThrAsnVal
CACCGCCAGGTCGTGGAGGTCACCGTGGGCCTGCAGCTGATACAGCTCATCAATGTGGATGAAGTAAATCAGATCGTGACAACCAATGTG
      80            100            120            140            160
                  60                              80
ArgLeuLysGlnGlnTrpValAspTyrAsnLeuLysTrpAsnProAspAspTyrGlyGlyValLysLysIleHisIleProSerGluLys
CGTCTGAAACAGCAATGGGTGGATTACAACCTAAAATGGAATCCAGATGACTATGGCGGTGTGAAAAAAATTCACATTCCTTCAGAAAAG
            180            200            220            240
                              100
IleTrpArgProAspLeuValLeuTyrAsnAsnAlaAspGlyAspPheAlaIleValLysPheThrLysValLeuLeuGlnTyrThrGly
ATCTGGCGCCCAGACCTTGTTCTCTATAACAATGCAGATGGTGACTTTGCTATTGTCAAGTTCACCAAAGTGCTCCTGCAGTACACTGGC
      260            280            300            340
        120                              140
HisIleThrTrpThrProProAlaIlePheLysSerTyrCysGluIleIleValThrHisPheProPheAspGluGlnAsnCysSerMet
CACATCACGTGGACACCTCCAGCCATCTTTAAAAGCTACTGTGAGATCATCGTCACCCACTTTCCCTTTGATGAACAGAACTGCAGCATG
      360            380            400            420
                              160
LysLeuGlyThrTrpThrTyrAspGlySerValValAlaIleAsnProGluSerAspGlnProAspLeuSerAsnPheMetGluSerGly
AAGCTGGGCACCTGGACCTACGACGGCTCTGTCGTGGCCATCAACCCGGAAAGCGACCAGCCAGACCTGAGCAACTTCATGGAGAGCGGG
      440            460            480            500            520
        180                                          200
GluTrpValIleLysGluSerArgGlyTrpLysHisSerValThrTyrSerCysCysProAspThrProTyrLeuAspIleThrTyrHis
GAGTGGGTGATCAAGGAGTCCCGGGGCTGGAAGCACTCCGTGACCTATTCCTGCTGCCCCGACACCCCCTACCTGGACATCACCTACCAC
      540            560            580            600
                              220
PheValMetGlnArgLeuProLeuTyrPheIleValAsnValIleIleProCysLeuLeuPheSerPheLeuThrGlyLeuValPheTyr
TTCGTCATGCAGCGCCTGCCCCTCTACTTCATCGTCAACGTCATCATCCCCTGCCTGCTCTTCTCCTTCTTAACTGGCCTGGTATTCTAC
      620            640            660            680            700
        240                              260
LeuProThrAspSerGlyGluLysMetThrLeuSerIleSerValLeuLeuSerLeuThrValPheLeuLeuValIleValGluLeuIle
CTGCCCACAGACTCAGGCAGAAGATGACTCTGAGCATCTCTGTCTTACTGTCTTTGACTGTGTGTTCCTTCTGGTCATCGTGGAGCTGATC
            740            760            780
                              280
ProSerThrSerSerAlaValProLeuIleGlyLysTyrMetLeuPheThrMetValPheValIleAlaSerIleIleIleThrValIle
CCCTCCACGTCCAGTGCTGTGCCCTTGATTGGAAAATACATGCTGTTCACCATGGTGTTCGTCATTGCCTCCATCATCATCACTGTCATC
      800            820            840            860            880
        300                                          320
ValIleAsnThrHisHisArgSerProSerThrHisValMetProAsnTrpValArgLysValPheIleAspThrIleProAsnIleMet
GTCATCAACACACACCACCGCTCACCCAGCACCCATGTCATGCCCAACTGGGTGCGGAAGGTTTTTATCGACACTATCCCAAATATCATG
            900            920            940            960
                              340
PhePheSerThrMetLysArgProSerArgGluLysGlnAspLysLysIlePheThrGluAspIleAspIleSerAspIleSerGlyLys
TTTTTCTCCACAATGAAAAGACCATCCAGAGAAAAGCAAGACAAAAAGATTTTTACAGAAGACATTGATATCTCTGACATTTCTGGAAAG
      980            1000            1020            1040            1060
        360                                          380
ProGlyProProProMetGlyPhe     ProLeuIleLysHisProGluValLysSerAlaIleGluGlyIleLysTyrIleAlaGlu
CCAGGGGCTCCACCACCCATGGGCTTC   CCCCTGATCAAACACCCCGAGGTGAAAAGTGCCATCGAGGGCATCAAGTACATCGCAGAG
            1080            1100            1120            1140
        420
ThrMetLysSerAspGlnGluSerAsnAsnAlaAlaAlaGluTrpLysTyrValAlaMetValMetAspHisIleLeuLeuGlyValPhe
ACCATGAAGTCAGACCAGGAGTCTAACAATGCGGCGGCAGAGTGGAAGTACGTTGCAATGGTGATGGACCACATACTCCTCGGAGTCTTC
      1160            1180            1200            1220            1240
MetLeuValCysIleIleGlyThrLeuSerValPheAlaGlyArgLeuIleGluLeuAsnGlnGlnGlyEnd
ATGCTTGTTTGCATCATCGGAACCCTA  GTTTGCAGGTCGACTCATTGAATTAAATCAGCAAGGATGAGCAGAAAATGAGCTGAGC
            1260            1280            1300            1320
TTAGCTCTGCCCTGGAACCTACCAGAGCAGAGAAGGGCAGGAGAGGAAGATTTGTCTACTTGCTCCACTCGCACTTATCAAACGTGTTAT
      1340            1360            1380            1400            1420
ATTCCATACTTATTATTGATGATAAGATTTACCTTTATGTAAGTTTATGGCCTTGAAGTGTTTTCATATTGCTTCTCCCTTTAGTTCTGC
            1440            1460            1480            1500
TGTCTCCCTGAAGAGTGAACCCTCTTTAGTAAATGAAACTAATCACTAAAAAAAAAAAAAAAAAAAA
      1520            1540            1560
```

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Figure 10. Comparison of the amino acid sequence deduced for α subunits of receptors from TE671 cells with the sequences deduced for α subunits of muscle type nicotinic receptors from other species. Reproduced from reference 23.

Figure 11. Nucleotide and deduced amino acid sequence of a TE671 cDNA clone coding for the δ subunit of receptor from TE671 cells. Reproduced from reference 23.

Figure 12. Comparison of the amino acid sequence deduced for δ subunits of receptors from TE671 with the sequences of δ subunits from nicotinic receptors from several species. Reproduced from reference 23.
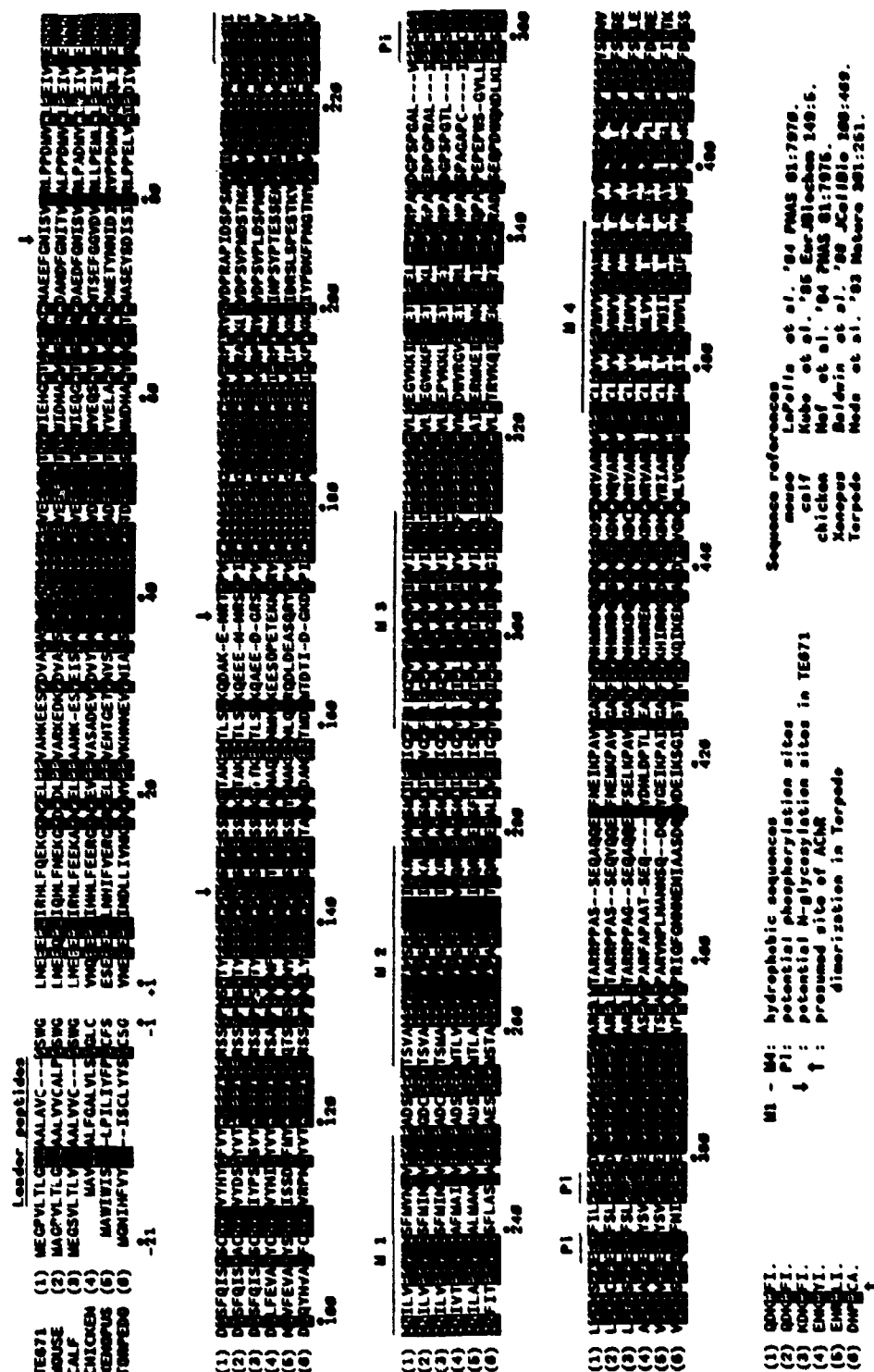
Figure 13. Expression of nicotinic receptors by TE671 cells is affected by nicotine, dexamethasone, CGRP and forskolin. On day zero 1×10^5 cells were plated in each 3.5-cm dish. On day 2 media were supplemented as indicated. On day 4 carbamylcholine-induced $^{86}Rb^+$ influx was measured on triplicate cultures. Background for each culture condition (about 500 cpm) was subtracted. Northern blots using equal amounts of total RNA from sister cultures were successively probed with ^{32}P-labeled mouse α, β, and γ and human δ cDNAs. ^{125}I-α-bungarotoxin binding was measured in other sister cultures. In the table at the bottom of the figure, arrows facing upward imply increase, horizontal arrows imply no significant change, and arrows pointing downward indicate decrease. mRNA blots were not scanned for quantification, so these results should be considered a preliminary overview. Reproduced from reference 23.
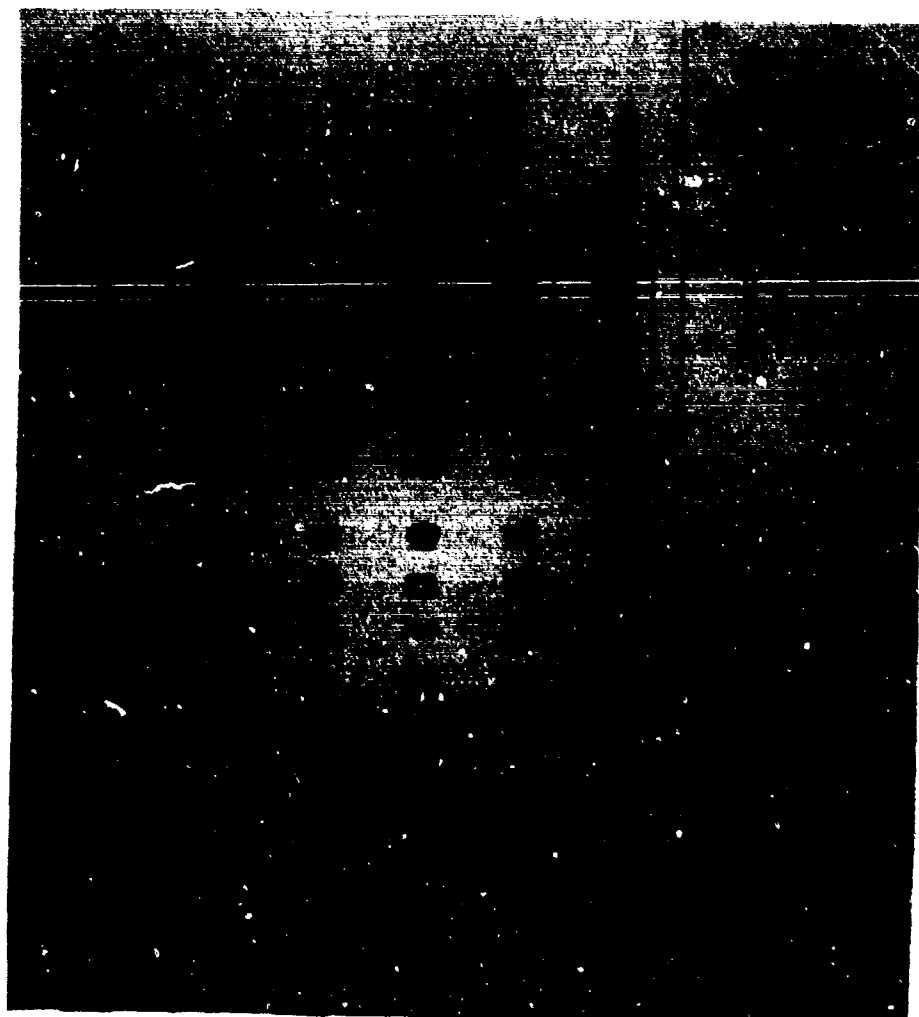

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Figure 14. Forskolin affects cell division and morphology of TE671 cells. Equal amounts of cells were grown for 48 hours in Iscove's medium plus 5% fetal bovine serum plus (B,D) or minus (A,C) 20 μM forskolin. Note that in the presence of forskolin, cell density is reduced and extension of processes by the cells is greatly increased. Reproduced from reference 23.
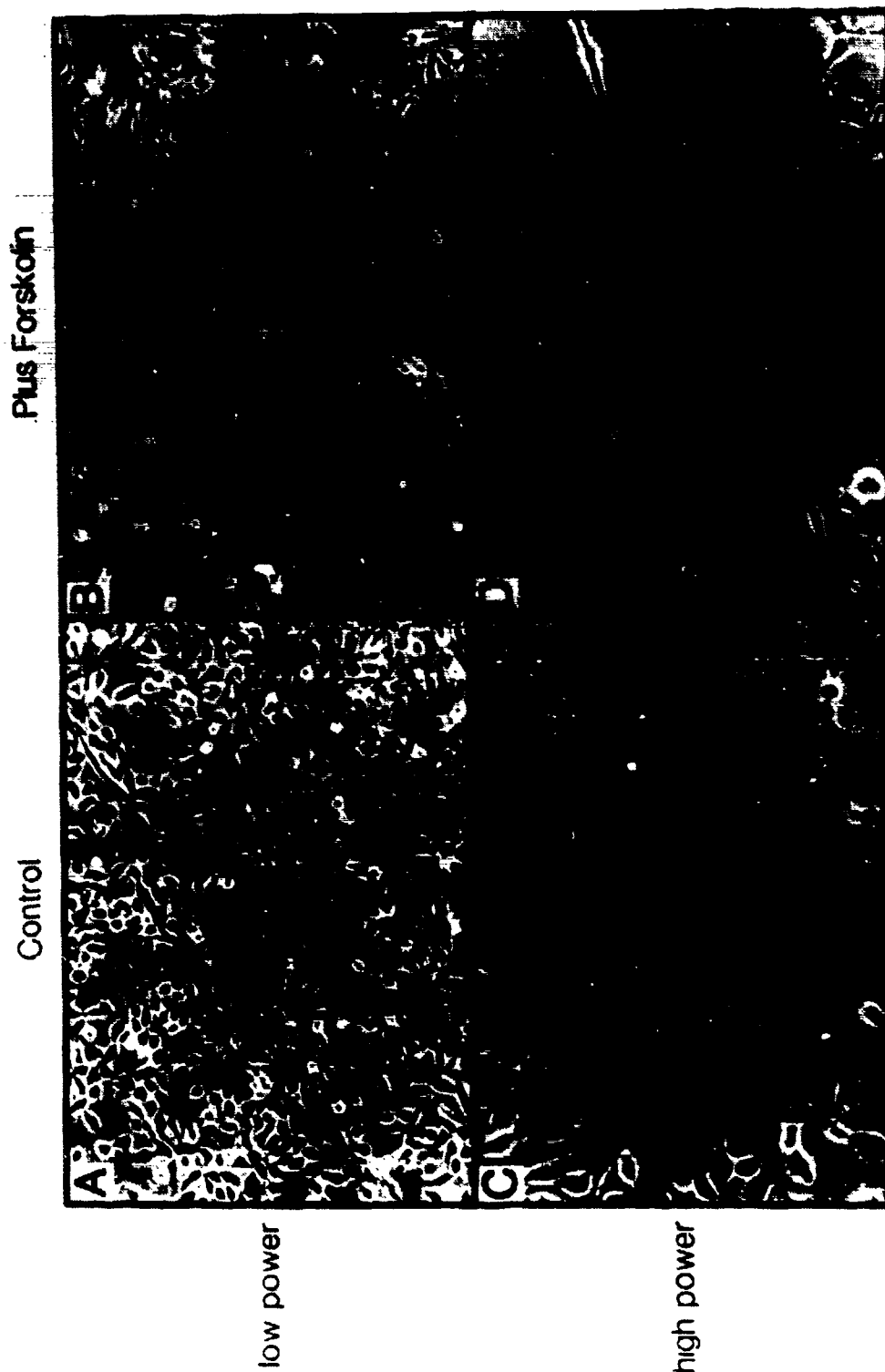
Figure 15. Structure and N-terminal sequences of chicken and rat brain nicotinic receptors. The cDNA for this acetylcholine-binding subunit of receptors from rat brain has been termed 'α4.'[11,12] The cDNA for this structural subunit has been termed 'β2.'[14,15]

Amino Terminal Sequence

ACh Binding Subunit:
cDNA — HIETRAHAEERLLKRLF Rat
Protein — XIETXAXAEEXLLKXLF Rat

Structural Subunit:
cDNA — TDTEERLVEYLLDP Chicken
Protein — TDXEEXLVEYLLDP Chicken
Protein — TDTEEXLVEXLXDX Rat

Purified AChR from Brain — Rat, Chicken

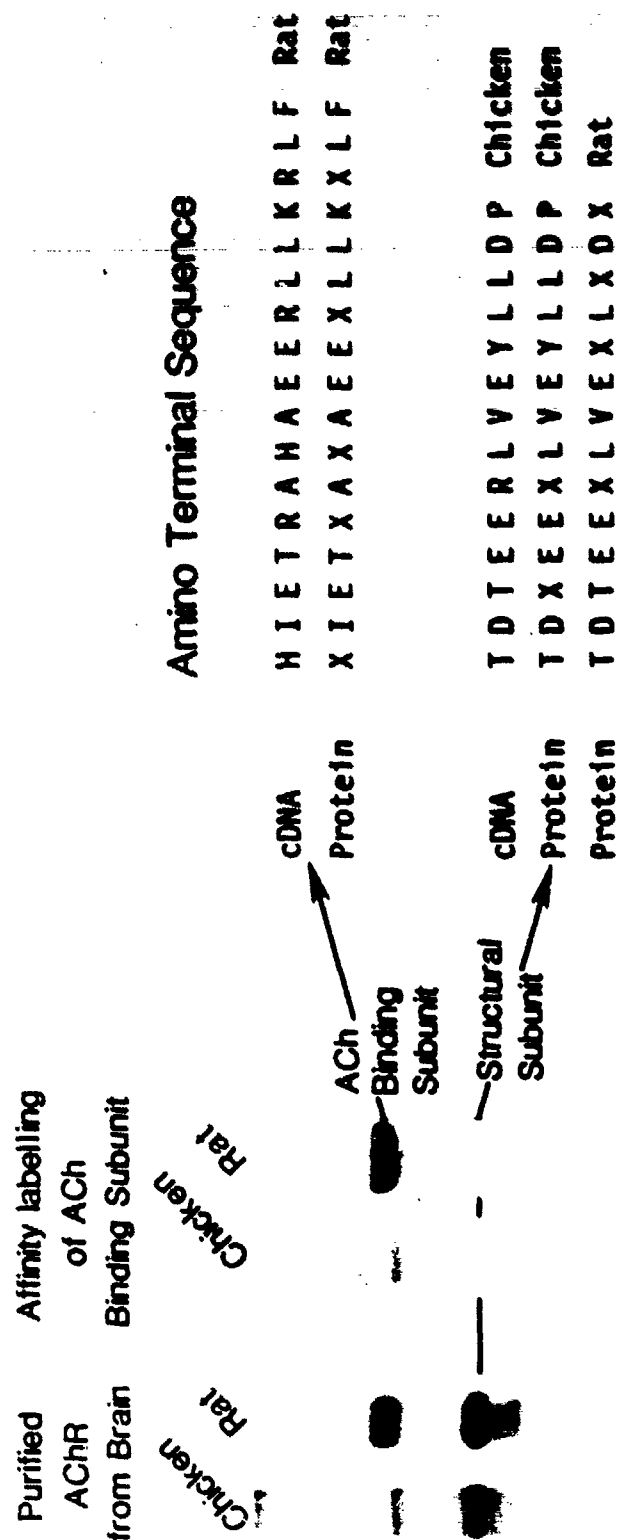Affinity labelling of ACh Binding Subunit — Chicken, Rat

Figure 16. Nucleic acid and deduced protein sequences of structural subunit of nicotinic receptors from chicken brain. Reproduced from reference 15.

```
                                            MetAlaLeuLeuArgValLeuCysLeuLeuAlaAlaLeuArgArgSerLeuCys
GJCAGCGGCACCGGgArCGGCACCGGCGGAGGCAGCGGGCCGCCCCCcATGGCGCTGCTCCGCGTCCTCTGCCTCCTCGCCGCGCTCCGACGCAGTCTGTGC
 88           -88          -68           -48           ~28          8

ThrAspThrGluGluArgLeuValGluTyrLeuLeuAspProThrArgTyrAsnLysLeuIleArgProAlaThrAsnGlySerGlnLeuValThrValGln
ACGGACACGGAGGAGCGCCTGGTGGAATACCTCCTGGACCCCACACGCTACAACAAACTCATCCGCCCGGCCACCAACGGCTCTCAGCTCGTCACCGTGCAG
1            28           48           68           88           188

LeuMetValSerLeuAlaGlnLeuIleSerValHisGluArgGluGlnIleMetThrThrAsnValTrpLeuThrGlnGluTrpGluAspTyrArgLeuThr
CTCATGGTGTCACTGGCACAGCTCATCAGTGTGCACGAGCGGGAGCAGATCATGACCACCAACGTGTGGCTGACCCAGGAATGGGAGGACTATCGCCTCACC
        128          148          168          188          288

TrpLysProGluAspPheAspAsnMetLysLysValArgLeuProSerLysHisIleTrpLeuProAspValValLeuTyrAsnAsnAlaAspGlyMetTyr
TGGAAGCCGGAGGACTTCGACAACATGAAGAAGGTCCGCCTGCCCTCCAAGCACATCTGGCTGCCTGATGTGGTACTCTACAACAACGCCGACGGGATGTAC
        228          248          268          288          388

GluValSerPheTyrSerAsnAlaValIleSerTyrAspGlySerIlePheTrpLeuProProAlaIleTyrLysSerAlaCysLysIleGluValLysHis
GAGGTCTCCTTCTACTCCAACGCCGTCATCTCCTACGACGGCAGCATCTTCTGGCTGCCCCCCGCCATCTACAAGAGCGCGTGCAAGATCGAGGTGAAGCAT
        328          348          368          388          4C8

PheProPheAspGlnGlnAsnCysThrMetLysPheArgSerTrpThrTyrAspArgThrGluIleAspLeuValLeuLysSerGluValAlaSerLeuAsp
TTCCCCTTCGACCAACAAAACTGCACCATGAAGTTCCGTTCCTGGACCTACGACCGCACTGAGATCGACCTGGTGCTGAAGAGCGAGGTGGCCAGCCTGGAT
        428          448          468          488          588

AspPheThrProSerGlyGluTrpAspIleValAlaLeuProGlyArgArgAsnGluAsnProAspAspSerThrTyrValAspIleThrTyrAspPheIle
GACTTCACGCCCAGCGGCGAGTGGGACATCGTGGCGCTGCCGGGACGGCGCAACGAGAACCCGGACGACTCCACCTACGTGGACATCACCTACGACTTCATC
        528          548          568          588          688

IleArgArgLysProLeuPheTyrThrIleAsnLeuIleIleProCysIleLeuIleThrSerLeuAlaIleLeuValPheTyrLeuProSerAspCysGly
ATCCGGCGCAAGCCGCTCTTCTACACCATCAACCTCATCATCCCCTGCATCCTCATCACCTCCCTGGCCATCCTCGTCTTCTACCTCCCCTCCGACTGCGGA
        628          648          668          688          788

GluLysMetThrLeuCysIleSerValLeuLeuAlaLeuThrValPheLeuLeuLeuIleSerLysIleValProProThrSerLeuAspValProLeuVal
GAGAAGATGACGCTCTGCATCTCCGTCCTGCTCGCCCTCACCGTCTTCCTGCTGCTCATCTCCAAGATCGTGCCGCCCACCTCGCTGGACGTGCCGCTGGTG
        728          748          768          788          888

GlyLysTyrLeuMetPheThrMetValLeuValThrPheSerIleValThrSerValCysValLeuAsnValHisHisArgSerProThrThrHisThrMet
GGCAAGTACCTCATGTTCACCATGGTGCTGGTGACCTTCTCCATCGTCACCAGCGTCTGCGTGCTCAACGTGCACCACCGCTCGCCCACCACTCACACAATG
        828          848          868          888          988          9

ProProTrpValArgThrLeuPheLeuArgLysLeuProAlaLeuLeuPheMetLysGlnProGlnGlnAsnCysAlaArgGlnArgLeuArgGlnArgArg
CCGCCCTGGGTGCGCACCCTTTTCCTCCGCAAGCTCCCCGGCGCTGCTCTTCATGAAGCAGCCGCAGCAGAACTGCGCGAGGCAACGCCTGCGCCAGCGTCGC
28           948          968          988          1888         1828

GlnThrGlnGluArgAlaAlaAlaAlaThrLeuPheLeuArgAlaGlyAlaArgAlaCysAlaCysTyrAlaAsnProGlyAlaAlaLysAlaGluGlyLeu
CAAACCCAGGAGCGTGCCGCAGCTGCCACGCTTTTCCTCCGGGCCGGTGCTCGTGCCTGCGCCTGCTATGCCAACCCTGGGGCTGCCAAAGCCGAGGGGCTC
        1848         1868         1888         1188         1128

AsnGlyTyrArgGluArgGlnGlyGlnGlyProAspProProAlaProCysGlyCysGlyLeuGluGluAlaValGluGlyValArgPheIleAlaAspHis
AATGGTTACCGGGAGCGGCAGGGGCAGGGGCCGGATCCCCCGGCACCGTGCGGCTGCGGGCTGGAGGAGGCGGTGGAGGGAGTGCGCTTCATTGCAGACCAC
        1148         1168         1188         1288         1228

MetArgSerGluAspAspAspGlnSerValSerGluAspTrpLysTyrValAlaMetValIleAspArgLeuPheLeuTrpIlePheValPheValCysVal
ATGCGCAGCGAGGACGATGACCAGAGTGTGAGCGAGGACTGGAAGTACGTGGCCATGGTCATCGACCGCCTCTTCTTATGGATCTTCGTCTTCGTCTGCGTC
        1248         1268         1288         1388         1328

PheGlyThrValGlyMetPheLeuGlnProLeuPheGlnAsnTyrAlaThrAsnSerLeuLeuGlnLeuGlyGlnGlyThrProThrSerLysEnd
TTCGGCACCGTCGGGATGTTCCTGCAGCCCCTCTTCCAGAACTACGCCACCAATTCCCTGCTGCAGCTCGGCCAGGGCACCCCCACCTCCAAATAGTGCCGG
        1348         1368         1388         1488         1428

GGGAGGGGGGGGGGGGGGGGUGGGGAAGGAGGGGGGGACCACAGGGGACGGTGGGGTTGTGCTCTCACCCCCCAGGGCGGGCAGAGCTGGGGCAGCCACTGCAA
        1448         1468         1488         1588         1528

ACCAATAAATAACTCACACAGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
        1548         1568         1588         1688
```

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Figure 17. Comparison of the amino acid sequence for the structural subunit of nicotinic receptors from chicken brain with the sequences of putative acetylcholine-binding subunits of receptors from rat brain and muscle reported by Boulter et al.[13] Note stretches of sequence identity throughout, which identify these subunits as members of the nicotinic receptor family. Note that the putative transmembrane domains M1, M2, and M3 are highly conserved. By contrast, note the putative cytoplasmic domain unique to each subunit. Reproduced from reference 15.

Figure 18. Possible neuronal nicotinic receptor subunit stoichiometries. Note that if the neuronal receptor with only two kinds of subunits were to preserve the fivefold symmetry of muscle acetylcholine receptors (with two acetylcholine-binding subunits and three structural subunits), then identical subunits would have to specifically associate both with themselves and with another subunit. Reproduced from reference 2.
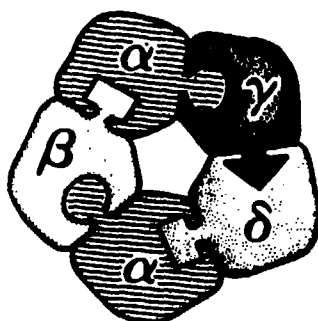
Muscle-type AChR

$$\alpha_2 \beta \gamma \delta$$
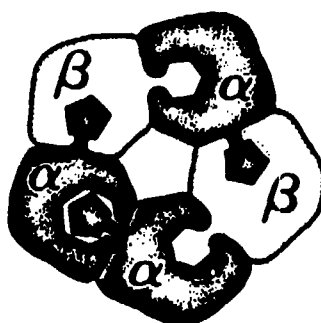
Neuronal AChR

$$\alpha_3 \beta_2$$
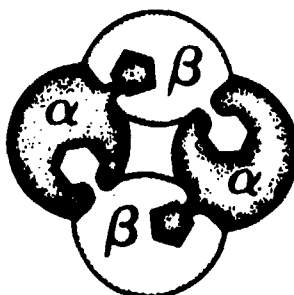
Neuronal AChR

$$\alpha_2 \beta_2$$

Figure 19. The distribution of ^{125}I-mAb 270 immunolabeling in a rostro-caudal (A-P) series of sections through the rat central nervous system. Virtually no labeling was observed when the sections were coincubated in 400 nM cold mAb 270. Adjacent Nissl-stained sections were used to identify labeled structures. Reproduced from reference 52.

Figure 20. Structural characterization of nicotinic receptors immunoaffinity purified from bovine brain using mAb 295. A, Silver-stained SDS acrylamide gels. B, Autoradiogram of ^{125}I-labeled purified receptor resolved on an SDS acrylamide gel. C, Western blot using mAb 268 to the structural subunit of receptor from chicken brain. D, Specific affinity labeling of the acetylcholine-binding subunit with [^{3}H]MBTA after reduction. Reproduced from reference 21.
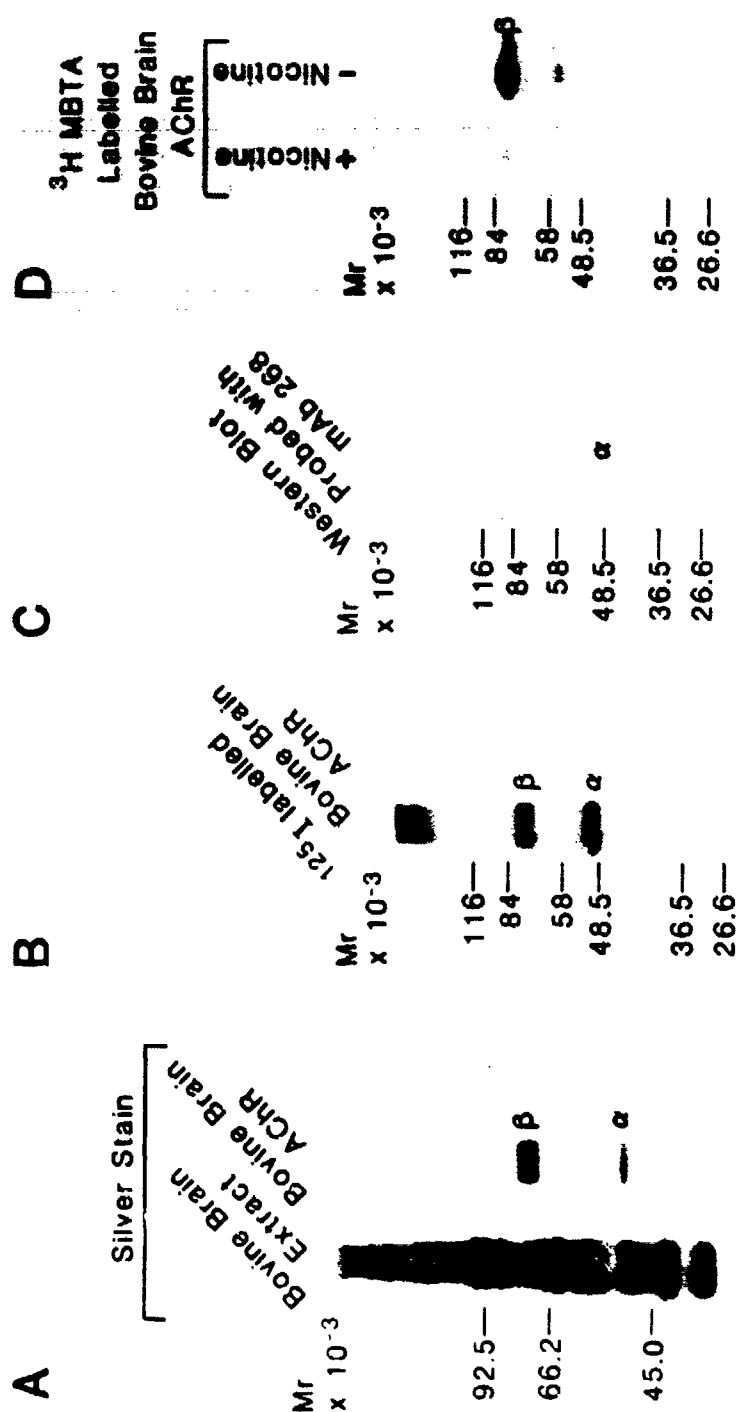

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Figure 21. Sucrose gradient analysis of nicotinic receptors from bovine brain (A) and human brain (B). Reproduced from reference 21.
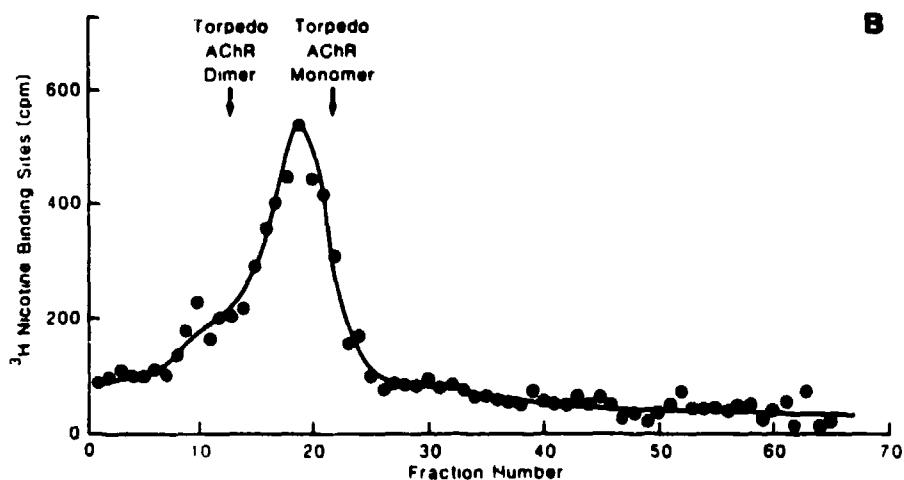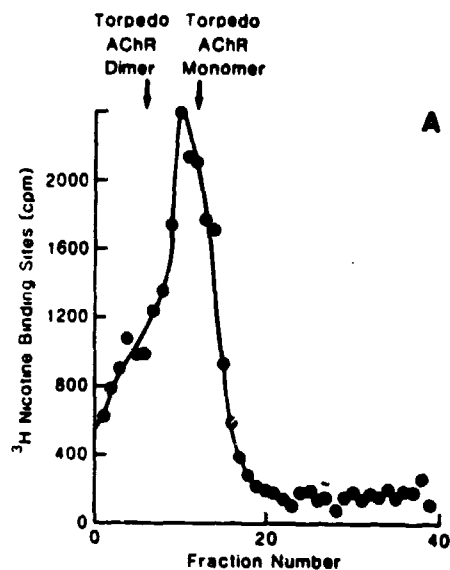
Figure 22. Immunological distinction of nicotinic receptors from human brain and muscle. mAB 290 is a monoclonal antibody to nicotinic receptors (AChR) from rat brains, which crossreacts with nicotinic receptors from human brain but not human muscle. Antiserum to nicotinic receptors purified from rat brains also failed to crossreact with receptors from muscle or TE671 cells. Myasethenia gravis (M.G.) patient autoantibodies to human muscle nicotinic receptors react with receptors from muscle and TE671 cells, but not from human brain. Reproduced from reference 20.
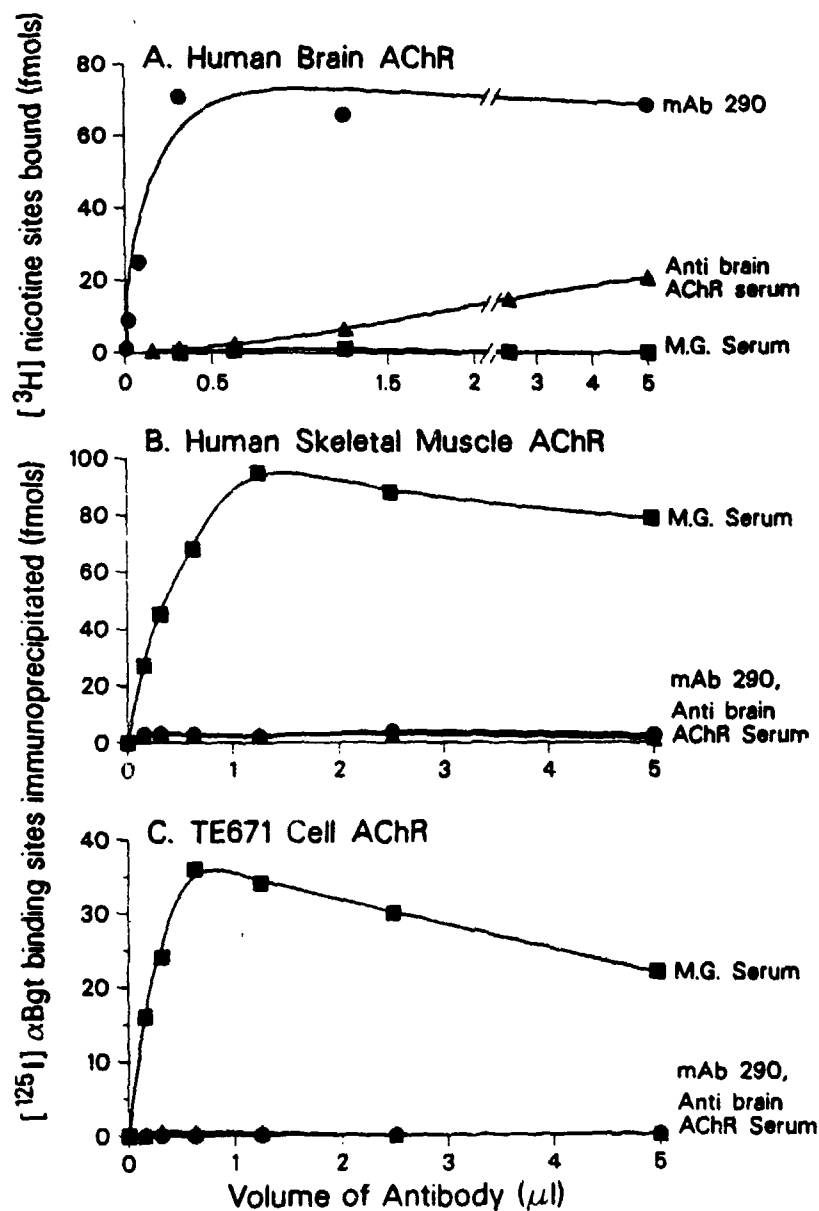
Figure 23. Binding of [³H]nicotine to receptors from bovine brain (A) and human brain (B). Receptors in Triton X-100 extracts were immobilized on mAb 290 coupled to goat anti-rat IgG Sepharose. Reproduced from reference 21.
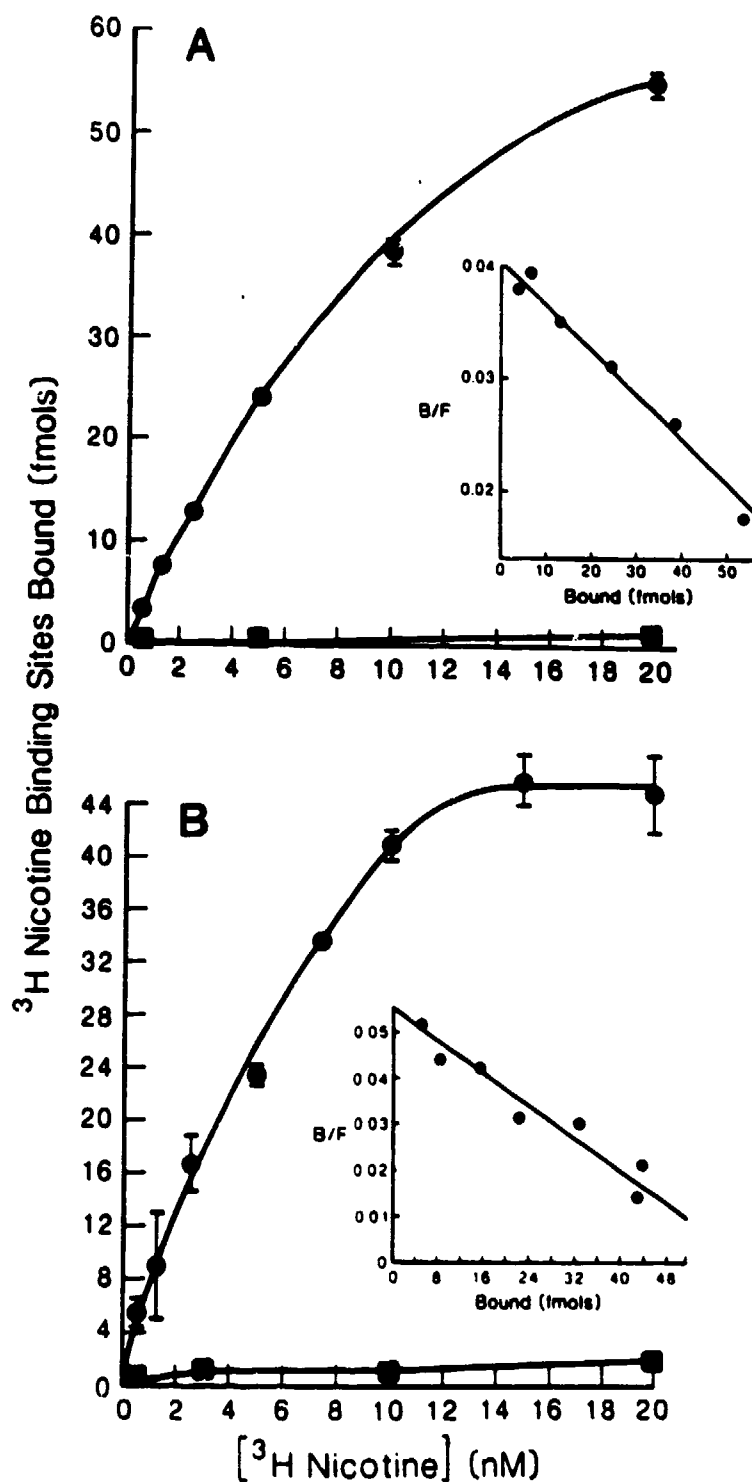
Figure 24. Affinity labeling of nicotine receptors from bovine brain (A) and human brain (B) with BAC (●) and MBTA (▲). BAC was 10^3-fold more effective. Receptors in Triton X-100 extracts were immobilized on mAb 290 coupled to goat anti-rat IgG Sepharose, reduced with 1 mM dithiothreitol, then labeled with BAC or MBTA. Following re-oxidation with 0.1 mM dithiobis (2-nitrobenzoic acid), binding of 10 nM [^{3}H]nicotine was assayed. Reproduced from reference 21.
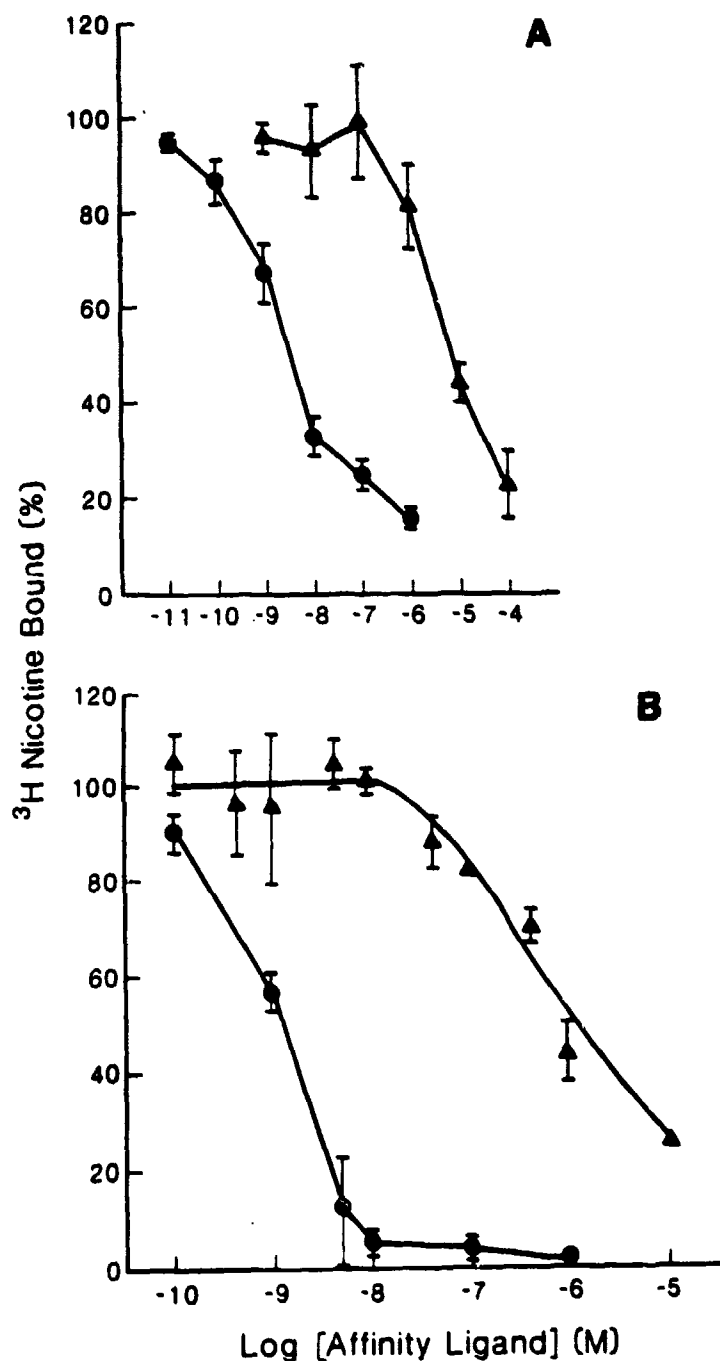
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